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(54) Title: AGENTS AND METHODS FOR BINDING TO ELAM-1

(57) Abstract

Identification of the sialyl Lewis X determinant as the determinant responsible for ELAM-1-dependent adhesion of neutrophils to endothelium has resulted in the development of a number of agents for binding to ELAM-1 and methods for treating ELAM-1 mediated diseases.

Description

Agents and Methods for Binding to ELAM-1

The present invention was achieved in connection with NIH Contract No. R01-CA49256, and the government may have some rights to the present invention.

Technical Field

The present invention relates to compounds and methods for binding to ELAM-1.

Background Art

The LEC-CAM/SELECTIN family of cell adhesion molecules mediates adhesive interactions between circulating leukocytes and the vascular endothelium (Stoolman, Cell, vol. 56, pp. 907-910 (1989); and Geng et al., Nature, vol. 343, pp. 757-760 (1990)). These molecules participate in the recruitment of neutrophils and monocytes to inflammatory lesions (Carlos and Harlan, Immunol. Rev., vol. 114, pp. 1-24 (1990); and Jutila et al., Transplantation, vol. 48, pp. 727-731 (1989)), in the adhesion of activated platelets to leukocytes (Larsen et al., Cell, vol. 59, pp. 305-312 (1989)), in the recirculation of normal lymphocytes through the lymphoid system (Yednock et al., Adv. Immunol., vol. 44, pp. 313-378 (1989)), and in the hematogenous dissemination of lymphoid malignancies (Bartgatze et al., J. Exp. Med., vol. 166, pp. 1125-1131 (1987)). Three structurally related receptors have thus far been identified - ELAM-1 (Bevilacqua et al., Science, vol. 243, pp. 1160-1165 (1989)), GMP-140/PADGEM (Bonfanti et al., Blood, vol. 73, pp. 1109-1112 (1989); and Johnston et al., Cell, vol. 56, pp.

1033-1044 (1989)), and LEC-CAM1 (consensus term referring to the antigen expressing the Mel14 epitope in the mouse (Bowen et al., J. Cell. Biol., vol. 109, pp. 421-427 (1989); Lasky et al., Cell, vol. 56, pp. 1045-1055 (1989)) and its human homologues (Leu8/TQ1; Camerini et al., Nature, vol. 342, pp. 78-82 (1989); LAM, Tedder et al., J. Exp. Med., vol. 170, pp. 123-133 (1989); and DREG, Kishimoto et al., Proc. Natl. Acad. Sci., vol. 87, pp. 2244-2248 (1990))). The N-terminal domains of these molecules are homologous to one another and to a variety of calcium-dependent carbohydrate recognition domains (CRDs) containing a structural motif originally described by Drickamer and colleagues (Drickamer, J. Biol. Chem., vol. 263, pp. 9552-9560 (1988)). The CRD of LEC-CAM1 mediates adhesion to high endothelial venules (HEV) in vitro (Geoffroy and Rosen, J. Cell Biol., vol. 109, pp. 2463-2470 (1989); and Yednock and Rosen, Adv. Immunol., vol. 44, pp. 313-378 (1989)) and appears to initiate lymphocyte recirculation through binding to one or more sialylated ligands on the HEV in vivo (Rosen et al., Science, vol. 228, pp. 1005-1007 (1985); and Rosen et al., J. Immunol., vol. 142, pp. 1895-1902 (1989)).

ELAM-1 mediates the adhesion of blood leukocytes to inflammed endothelium. It is induced by a variety of soluble mediators of inflammation including but not limited to the immune cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF), and the bacterial endotoxins (LPS). The immune cytokines are elaborated by monocytes, keratinocytes and possibly fibroblasts in response to inflammatory stimuli such as infection, autoimmune response and tissue necrosis resulting from ischemic injury. LPS refers to a heterogeneous group of lipopolysaccharides derived from the cell walls of gram negative bacteria. These broad spectrum inflammatory

stimuli contribute to the vascular complications of bacterial sepsis including the acute respiratory distress syndrome (ARDS) and shock. The expression of ELAM-1 on the microvasculature in such diseases as psoriasis, rheumatoid arthritis and delayed hypersensitivity reactions further indicates a role for this molecule in the development of pathologic inflammation. In brief, ELAM-1 dependent attachment of leukocytes to the endothelium may contribute to diverse illnesses in which the inflammatory response leads to tissue damage including (1) the acute respiratory distress syndrome (ARDS); (2) rheumatoid arthritis (Grober, J. et al., submitted; Koch, A.E. 1990, Am. J. Pathol., in press); (3) Psoriasis and hypersensitivity disorders of the skin (Nickoloff, B. et al., submitted); (4) reperfusion injury resulting from myocardial infarction and (5) hypovolemic shock.

Accordingly, there is interest in molecules which can bind to ELAM-1 and block the attachment of leukocytes to human endothelial cells expressing ELAM-1. These molecules may be used as therapeutic agents for the treatment of diseases mediated by the expression of ELAM-1 on the surface of human endothelial cells or other cells. In addition, it is desirable to enable cells, which do not normally possess the necessary determinant for recognizing ELAM-1, to express such determinant and thus adhere to human endothelial cells or other cells expressing ELAM-1 on their surfaces. In this way, cells, which either naturally express or express as a result of recombinant DNA engineering a particular product, may be directed to the region of the vasculature in which the endothelial cells are expressing ELAM-1, so as to specifically deliver the particular product to the stressed region of the

vasculature. Such cells could also be used as hosts for the production of recombinant glycoproteins, or other glycoconjugate-containing molecules, that contain the ligand(s) for ELAM-1, by virtue of the fact that they maintain the necessary biochemical machinery necessary for the synthesis of such ligands. It is also desirable to prepare molecules in which a drug or therapeutic agent is covalently linked to a molecule which recognizes the ELAM-1 binding site and thus, are capable of directing the delivery of the drug or therapeutic agent to the stressed region of the vasculature.

However, until now, the determinant on the surface of neutrophils responsible for the recognition and adhesion to ELAM-1 has remained unknown, and a process capable of generating, in quantity, high affinity ligands for ELAM-1 has not been available.

Disclosure of the Invention

Accordingly, it is one object of the present invention to provide a method for treating diseases mediated by the elaboration of ELAM-1 on endothelial cells.

It is another object to provide a method for treating ischemia following reperfusion.

It is another object to provide a method for treating inflammation.

It is another object to provide a method for treating infarction of myocardial tissue.

It is another object to provide a method for treating Adult Respiratory Distress Syndrome (ARDS).

It is another object to provide a method for treating rheumatoid arthritis.

It is another object to provide a method for treating psoriasis and hypersensitivity diseases of the skin.

It is another object to provide molecules which bind to ELAM-1 and thus retard the adhesion of leukocytes to endothelial cells expressing ELAM-1.

It is another object to provide compounds in which a drug or therapeutic agent is covalently bonded to a molecule which binds to ELAM-1.

It is another object to provide transfected cells, which do not normally express the ability to adhere to endothelial cells expressing ELAM-1, which as a result of their being transfected possess the ability to adhere to endothelial cells expressing ELAM-1.

It is another object to provide pharmaceutical compositions for treating diseases mediated by the elaboration of ELAM-1 on endothelial cells.

These and other objects, which will become apparent during the following detailed description, have been achieved by the inventors' discovery that expression of a family of $\alpha(2,3)$sialylated, $\alpha(1,3)$fucosylated lactosaminoglycans on a cell's surface results in ELAM-1 dependent endothelial

adhesion, and that compounds containing the sialyl Lewis X determinant bind to ELAM-1.

Brief Description of the Drawings

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying drawings, wherein:

Figure 1a illustrates the structures and biosynthesis of type II-based sialyl Lewis X and Lewis X molecules. Terminal galactose residues on neutral type II precursors may be substituted with α(2,3)-linked sialic acid via the action of an α(2,3)sialyltransferase (α(2,3)sialyl-T, Weinstein et al., J. Biol. Chem., vol. 257, pp. 13845-13853 (1982)). The subterminal GlcNAc residues on this sialylated precursor molecule, or on its neutral predecessor, may then each be substituted with α(1,3)-linked fucose residues to form the sialyl Lewis X (sLex) and Lewis X (Lex) determinants, respectively. This occurs via the action of α(1,3)fucosyltransferases that can operate exclusively on neutral precursors (e.g., Fuc-TI, Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987)), or that operate on both neutral and sialylated precursors (e.g., Fuc-TI, Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987)). R = glycoprotein or glycolipid moieties that may contain one or more additional lactosamine (Galβ1,4GlcNAcβ1,3) repeat units.

Figure 1b illustrates the structures of the type I-based sialyl Lewis a and Lewis a determinants. The sialyl Lewis a

(sLea) and Lewis a (Lea) molecules are thought to be constructed from sialylated and neutral type I precursors, respectively, by the action of α(1,4)fucosyltransferases, in a manner strictly analogous to the biosynthesis of the type II structures shown in Figure 1a (Hansson and Zopf, J. Biol. Chem., vol. 260, pp. 9388-9392 (1985)). R = glycoprotein or glycolipid moieties that display the type I precursor oligosaccharide.

Figure 2a illustrates the binding of HL-60 cell lines to human endothelial cell monolayers. HL-60 lines A and B were tested for adherence to TNFα-activated HUVEC, using an adhesion assay that isolates the ELAM-1-dependent component of this interaction (Method II in Examples, vide infra). Cells were allowed to adhere to TNFα-treated HUVEC monolayers, at 7-10°C, either in the presence of the anti-ELAM-1 antibody BB11 (solid bars), or in the presence of the control antibody IgG2b (open bars). Non-adherent cells were removed after 45 minutes; the fraction of remaining adherent cells are shown (% HL-60 Bound), and are mean determinations, +/- standard errors, representing a total of 14 (Hl-60 A) or 22 (HL-60 B) separate determinations from five independent experiments.

Figure 2b illustrates the results of the flow cytometry analysis of cell surface oligosaccharide determinants. HL-60 cell lines A and B were subjected to indirect immunofluorescence using the monoclonal antibodies directed against carbohydrate determinants detailed in the inset. Analyses were performed as described in the Examples, using a Coulter Epics V instrument equipped with a 3-decade scale.

Figure 3a illustrates the results of the flow cytometry

analysis of transfected COS-1 cells. Cells were transfected with two fucosyltransferase expression vectors (pCDM7-α(1,3/1,4)FT, or pCDNA1-α(1,3)FT; labeled, respectively, "α(1,3/1,4)FT", and "α(1,3)FT")), or with the control vector pCDM7. Transfected cells were then subjected to flow cytometry analysis with the monoclonal antibodies detailed in the inset, as described in the Examples. Between 26 and 31% of the cells transfected with pCDM7-α(1,3/1,4)FT or with pCDNA1-α(1,3)FT expressed, respectively, all four Le determinants, or Lex determinants only, relative to background staining with anti-H antibody (data not shown). The data presented here are the mean (linear) fluorescence intensities of the antigen-positive population of transfected cells.

Figure 3b illustrates the adhesion of transfected COS-1 cells to endothelial cell monolayers. Untransfected COS-1 cells (NIL), or COS-1 cells transfected with glycosyltransferase expression vectors or their control vectors, were radiolabelled with ^{51}Cr, harvested, and tested for adhesion on HUVEC monolayers using Method I, as detailed in the Examples. Transfected cells were applied to either TNFα-treated (+) or to untreated (-) HUVEC monolayers and allowed to adhere for 20 minutes prior to washing. Plasmids pCDM7-α(1,3/1,4)FT and pCDNA1-α(1,3)FT (labeled, respectively, "α(1,3/1,4)FT", and "α(1,3)FT") encode distinct α(1,3)fucosyltransferases as described in the Detailed Description. The corresponding control vectors for these plasmids (pCDM7 and pCDNA1) lack cDNA inserts. Plasmid pCDM7-αGT encodes a murine α(1,3)galactosyltransferase (Larsen et al., Proc. Natl. Acad. Sci. USA, vol. 86, pp. 8227-8231 (1989)). Plasmid pH3.4 encodes a human α(1,2)fucosyltransferase (Rajan et al., J. Biol. Chem., vol.

264, pp. 11158-11167 (1989)). COS-1 cells transfected with these latter two plasmids do not express Lex, sLex, Lea, or sLea determinants (data not shown). The adhesion of normal neutrophils (PMN) to HUVEC within the same experiment is also shown on a separately scaled ordinate. Error bars equal one standard deviation.

Figure 3c illustrates the specific anti-ELAM-1 blocking of adhesion of pCDM7-α(1,3/1,4)FT transfected cells to TNFα-treated HUVEC monolayers. ^{51}Cr-labeled COS-1 cells transfected with pCDM7-α(1,3/1,4)FT, or the pCDM7 control, were assessed in the adhesion assay as outlined in Figure 3c, except that the HUVEC monolayers had been pretreated with antibody directed against ELAM-1 (BB11), with an isotope control (IgG2b), or with antibodies against other endothelial-expressed polypeptides (anti-VCAM1 4B9, anti-ICAM1, and anti-HLA class 1), as described in the Examples. Error bars equal one standard deviation.

Figure 4a illustrates the results of the flow cytometry analysis of transfected Chinese hamster ovary cells. CHO cells stably transfected with the fucosyltransferase expression vector pCDM7-α(1,3/1,4)FT(CHO-FT) or with the control vector pCDM7 (CHO-V) were subjected to flow cytometry analysis with the monoclonal antibodies detailed in the figure and as described in the Examples. The data presented here are the mean fluorescence intensities of the entire population of these transfected cells; virtually 100% of the CHO-FT cells stain with anti-sLex and anti-Lex antibodies, but not with the other three antibodies (data not shown). Likewise, the entire population of CHO-V cells do not stain with any of the antibodies.

Figure 4b illustrates the time course of adhesion of CHO cells stably-transfected with pCDM7-α(1,3/1,4)FT(CHO-FT) or vector alone (CHO-V). Transfected cell lines were labeled with ^{51}Cr, harvested, and tested for adhesion on untreated (-) or TNFα-treated (+) HUVEC monolayers. Non-adherent cells were removed after the times shown, and adhesion was determined by Method I as described in the Examples. Error bars equal one standard deviation.

Figure 4c illustrates the specific anti-ELAM-1 blocking of adhesion of CHO-FT cells to TNFα-treated HUVEC monolayers. ^{51} Cr-labeled CHO-FT cells were subjected to adhesion for 10 minutes, as outlined in Figure 4b, except that the HUVEC monolayers had been pretreated with the anti-ELAM-1 antibody BB11, the isotype control IgG2b, or with antibodies against VCAM1 and HLA class 1 molecules, at the same concentrations used in Figure 3c. Pretreatment with anti-ICAM1 fails to inhibit binding to TNFα-treated HUVEC monolayers (data not shown). Error bars equal one standard deviation.

Best Mode for Carrying Out the Invention

Accordingly, one aspect of the present invention relates to molecules, which bind to ELAM-1 as expressed on the surface of human endothelial cells and thus, are able to inhibit or prevent the adhesion of leukocytes to endothelial cells expressing ELAM-1 on their surface.

It has now been demonstrated that expression of an α(1,3/1,4)fucosyltransferase results in the appearance of ligands for ELAM-1 on the surface of transfected COS-1 and CHO

cells. In COS-1 transfectants, the sialyl Lewis X (sLex), Lewis X (Lex), Lewis a (Lea) and sialyl Lewis a (sLea) oligosaccharides appeared at the surface while in CHO transfectants, only new sLex and Lex structures were detected. Thus, oligosaccharides terminating in the Lea and sLea structures are not necessary for expression of ELAM-1 dependent cell adhesion. Moreover, ELAM-1-dependent adhesion was not manifested either by COS-1 cells transfected with pCDNA1-α1,3FT, or by the variant of the HL-60 cell line, when expression of the Lex determinant occurs in the absence of the sLex structure. It is thus concluded that expression of α(1,3)fucosyltransferases capable of modifying acceptors containing α(2,3)sialic acid-substituted lactosaminoglycans is a critical step in the synthesis of the ligand(s) for ELAM-1. Therefore, one or more members of the family of sialylated, fucosylated lactosaminoglycans constructed by such enzymes are identified as the ligands for the CRD of ELAM-1.

The identities of the protein or lipid molecules that display the candidate oligosaccharide ligand(s) are as yet unknown. However, it is concluded that polylactosamine-type ganglioside precursors contained in normal myeloid cells (Fukuda et al., J. Biol. Chem., vol. 260, pp. 1067-1082 (1985)) are not essential to the generation of ELAM-1 binding activity, since the CHO host used for the stable transfection experiments does not construct such molecules (Smith et al., J. Biol. Chem., vol. 265, pp. 6225-6234 (1990)). As this observation demonstrates, the non-hematopoietic COS-1 and CHO cells are unlikely to express unique, myeloid-specific protein or lipid structures. Thus, it is concluded that the oligosaccharide moieties determined by the transfected cDNA are themselves sufficient to mediate ELAM-1 dependent

adhesion.

The sLex structure and its mono- and polyfucosylated analogues are examples of such molecules. Thus, one aspect of the present invention relates to molecules which are able to bind to ELAM-1 expressed on the surface of human endothelial cells and have the general formula (I):

$$NeuAc\alpha 2\rightarrow 3Gal\beta 1\rightarrow 4(Fuc\alpha 1\rightarrow 3)GlcNAc\alpha 1\rightarrow R \qquad (I)$$

wherein NeuAc represents a sialyl group (N-acetylneuraminyl group), Gal represents a D-galactosyl group, Fuc represents a L-fucosyl group, and GlcNAc represents an N-acetylglucosamine. The greek letter α indicates that the sugar moiety on the left hand side of the linkage is linked to the sugar moiety on the right hand side of the linkage by an α-linkage, and β indicates that the sugar moiety on the left hand side of the linkage is linked to the sugar moiety on the right hand side of the linkage by a β-linkage. The stereochemistry of the linkages is further defined by the number and arrow system, in which the first number indicates the position of left-hand sugar moiety involved in the linkage and the second number indicates the position of the right-hand sugar moiety involved in the linkage. It is to be understood that in formula (I), R is bonded directly to carbon atom number 1 of the GlcNAc group.

In formula (I), R may be OH, a protected hydroxy group, a C_{1-12} alkyl group, $-O-(CH_2)_n-CO_2R''$ (wherein R'' is C_{1-4} alkyl and n is 2 to 12), $-O-(CH_2)_n-CONHNH_2$ (n is 2 to 12), $-O-(CH_2)_nCON_3$ (n is 2 to 12), a lipid or glycolipid, a glycoconjugate consisting of a serine/threonine-linked oligosaccharide on a

protein or peptide, a glycoconjugate consisting of a free oligosaccharide derived by enzymatic or chemical hydrolysis from a serine/threonine-linkage to a protein or peptide, a glycoconjugate consisting of an asparagine-linked oligosaccharide on a protein or peptide, a glycoconjugate consisting of a free oligosaccharide derived by enzymatic or chemical hydrolysis from an asparagine linkage to a protein or peptide. Such glycoconjugates may include polylactosamine-type oligosaccharides whose internal GlcAc moieties may each be unsubstituted, or substituted with $\alpha(1,3)$fucose linkages. Such molecules include single (VIM-2, Macher, B.A. et al., J. Biol. Chem., vol. 263, pp. 10186-10191 (1988)) or multiple (FH4 and FH5, Fukushi, Y. et al., J. Biol. Chem., vol. 259, pp. 4681-4685 (1984); Fukushi, Y., et al., Cancer Res., vol. 45, pp. 3711-3717 (1985)) internal fucose residues in $\alpha(1,3)$-linkage to internal GlcNAc moieties. All of these may be synthesized with chemical and/or enzymatic methods, or may be purified from natural sources (human cells of the myeloid lineage, for example). However, that they represent part or all of the oligosaccharide ligand(s) for ELAM-1 has not been heretofore appreciated.

Suitable protecting groups for the protected hydroxy group of R include those disclosed in European Patent Application 184,162, which is incorporated herein by reference.

In another embodiment, R may represent a drug or a label linked to the molecule or a liposome encapsulating a drug or label. Examples of suitable drugs include drugs encapsulated in or otherwise incorporated into liposomes (antiinflammatory agents such as corticosteroids, cytokine antagonists,

antiinflammatory polypeptides including some interleukins, indomethacin, gold salts, cyclosporine, and antiinflammatory eicosanoids or enzymes that would increase the local concentrations of antiinflammatory eicosanoids; cytotoxic/chemotherapeutic agents that include adriamycin, doxorubicin, cisplatin, vincristine, cytarabine, bleomycin, amikacin, penicillin derivatives, Amphotericin B, or methotrexate, for example (Ranade, V.V., J. Clin. Pharmacol., vol. 29, pp. 685-694 (1989)). Other examples wherein R may represent a drug or a label linked to the molecule include proteins, peptides, or pharmaceutical compounds, including, by way of example, interleukins, antiinflammatory pharmaceuticals including corticosteroids, cytokine antagonists, indomethacin, and antiinflammatory eicosanoids or enzymes that would increase the local concentrations of antiinflammatory eicosanoids. Examples of suitable labels include drugs encapsulated in or otherwise incorporated into liposomes (gadolinium diethylenetriaminepentaacetic acid or other paramagnetic chelates for magnetic resonance imaging; technetium-99m and indium-111-NTA for scintigraphy; radiopaque [iodinated or brominated compounds] for computed tomography (Seltzer, S.E., Radiology, vol. 171, pp. 19-21 (1989)).

The present compounds may be prepared by conventional processes as described by Palcic et al, Carbohydrate Res., vol. 190, pp. 1-11 (1989); by Johnson et al, Biochem. Soc. Trans., vol. 13, pp. 1119-1120 (1985); Lemieux et al, J. Am. Chem. Soc., vol. 97, pp. 4056-4062, pp. 4063-4069, pp. 4069-4075, p. 4076 (1975); Inman et al, Immunochemistry, vol. 10, p. 165 (1973); Lemieux et al, Can. J. Biochem., vol. 55 (1977); which are incorporated herein by reference. Thus, the present compounds in which R is $-O-(CH_2)_nCO_2R''$ (in which R" is

a C_{1-4} alkyl group and n is an integer of from 2 to 12); $-O-(CH_2)_nCONHNH_2$ (n = 2 to 12); $-O-(CH_2)_nCON_3$ (n = 2 to 12); OH; or a C_{1-20} alkyl group may be easily prepared. The compounds in which R is an alkoxycarbonylalkyl group may be further derivatized as described by Lemieux et al, J. Am. Chem. Soc., vol. 97, pp. 4076-4083 (1975); Bundle et al, J. Immunol., vol. 129, pp. 678-682 (1982); Lemieux et al, Can. J. Biochem., vol. 55, pp. 507-512 (1977); and Lemieux et al, Biochemistry, vol. 20, pp. 199-205 (1981), which are incorporated herein by reference. Thus, by this method it is possible to prepare compounds in which R represents a group of the formula $-O(CH_2)_nCONH-P$ in which P represents a moiety derived from a protein, by reacting the compound in which R is $-O(CH_2)_nCON_3$ with a protein having free amino groups. Thus, in the compounds having R = $-O(CH_2)_nCOHN-P$, the "NH" portion of R originates as an amino group on the protein. Of course it is to be understood that this reaction may proceed with multiple additions when the protein, P, has more than one free amino group to yield compounds with a structure

$$(NeuAc\alpha 2\rightarrow 3Gal\beta 1\rightarrow 4(Fuc\alpha 1\rightarrow 3)GlcNAc\alpha 1\rightarrow O(CH_2)_nCONH)_x-P$$

in which x is limited by the number of free amino groups on the protein. In addition, the present compounds in which R includes a protein may be prepared by treating a glycoprotein which contains a suitable substrate with the appropriate enzyme, e.g., the fucosyl transferase encoded by the plasmid pCDM7-α(1,3/1,4FT).

The fact that compounds in which R is $-O(CH_2)_nCONHNH_2$ or $-O(CH_2)_nCON_3$ can be prepared also permits the synthesis of compounds in which R includes a moiety derived from a drug or a label. Thus, drugs or labels may be attached directly to

the R group by conventional techniques (see: Science, vol. 144, p. 1344 (1964), Immunochem., vol. 6, p. 53 (1969); and Peptides and Amino Acids, Benjamin, N.Y. (1966), which are incorporated herein by reference) or via any suitable bifunctional spacer. Suitable bifunctional spacers are disclosed in Haughland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Inc., Eugene, Oregon (1989) and Pierce Immunotechnology Catalog and Handbook, Pierce, Rockford, Il (1990), which are incorporated herein by reference.

Examples of suitable chemiluminescent labels are disclosed in German OLS No. 2,618,511, U.S. Patent 4,331,808, and Haughland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Inc., Eugene, Oregon (1989) which are incorporated herein by reference. Examples of suitable fluorescent labels are disclosed in Blecka et al, "Immunoassays in Therapeutic Drug Monitoring," Clinics in Laboratory Medicine, vol. 7, pp. 357-370 (1987) and Haughland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Inc., Eugene, Oregon (1989) which are incorporated herein by reference. Thus, the present compounds include those which may be used in chemiluminescent or fluorescent assays.

Suitable drugs include any which contain a reactive group which can couple directly with either $-O-(CH_2)_n-CONHNH_2$, $-O-(CH_2)_n-CON_3$, or a terminal group of an attached bivalent spacer. In addition, the drug may be one which has been derivatized to contain a reactive group for the purpose of linking, such as those described in U.S. Patent 4,331,808, incorporated herein by reference.

In addition, the present invention relates to liposomes which contain on their surface a liquid for ELAM-1, such as the sialyl Lewis X determinant. The production of liposomes is discussed in U.S. Patent Nos. 4,743,560 and 4,429,008; Batzri et al, Biochimica Biophysica Acta., vol. 298, pp. 1015-1019 (1973); Szoka et al, Proc. Natl. Acad. Sci. USA, vol. 75, pp. 4194-4198 (1978); Kojima et al, J. Biological Chem., vol. 264, pp. 20159-20162 (1989); and Eggens et al, J. Biological Chem., vol. 264, pp. 9476-9484 (1989); which are incorporated herein by reference. Thus, the present liposomes may be prepared by reacting liposomes which contain thiol reactive groups (see U.S. Patent 4,429,008) with a compound to introduce free amino groups on the surface of the liposome, and then reacting the liposome having free amino groups on the surface with a compound of formula (I) in which R is $-O-(CH_2)_n-CON_3$. For example, a liposome having maleimide groups on its surface may be reacted with a compound such as $H_2NCH_2CH_2SH$, to obtain a liposome having free $-NH_2$ groups. Alternatively, the present liposomes may be prepared by incorporating a molecule such as a ceramide which is bonded, either directly or through a suitable bifunctional spacer, to a ligand of ELAM-1, such as the sialyl Lewis X determinant (see: Eggens et al, J. Biological Chem., vol. 264, pp. 9476-9484 (1989); Batzri et al Biochim. Biophys. Acta., vol. 298, pp. 1015-1019 (1973); and Szoka et al, Proc. Natl. Acad. Sci., vol. 75, pp. 4194-4198 (1978)). The present liposomes may also contain drugs or other molecules as described in U.S. Patent Nos. 3,993,754 and 4,263,428, which are incorporated herein by reference. Additionally, the present liposomes may also encapsulate labels for NMR imaging or radionucleotide scanning. Suitable labels for use as radioactive diagnostics are disclosed in U.S. Patent 4,094,965 and Canadian Patent

Application 946,741, which are incorporated herein by reference. Suitable labels for NMR imaging are disclosed in French Patent Application 2,612,400, Eur. J. Med., vol. 24, pp. 241-247 (1989), UK Patent Application 2,214,507, and Radiology, vol. 175, pp. 483-488 (1990), which are incorporated herein by reference.

Another aspect of the present invention relates to transfected cells, which do not normally exhibit ELAM-1-dependent adhesion to endothelial cells, but which as a result of their transfection exhibit ELAM-1-dependent adherence to endothelial cells and a method for the production of such cells. Thus, such cells are those which either naturally or as a result of transfection produce a suitable substrate and have been transfected with either cDNA or genomic DNA encoding an enzyme capable of converting said suitable substrate into the sLex determinant.

Accordingly, the process for preparing the present cells involves:

(i) transfecting a starting cell, which does not express the sLex determinant and which does produce a substrate, with a DNA sequence coding for an enzyme which has the activity of converting the substrate to a molecule which contains the sLex determinant.

Thus, the starting cells may be any which do not express the sLex determinant but do produce a substrate which may be converted to a molecule containing the sLex determinant, by the enzyme encoded by the heterologous DNA which is introduced into the cells. Examples of suitable starting cells include

COS-1 and Chinese hamster ovary (CHO) line Ade⁻ C. The fact that pCDM7-α(1,3/1,4)FT transfection induces expression of ligands for ELAM-1 on non-hematopoietic cells indicates generalized distribution of suitable "acceptor" molecules for the α(1,3/1/4)FT. Therefore, it is reasonable to conclude that stable transfection of α(1,3/1,4)FT cDNA into (and expression in) a variety of normal cells and cell lines will result in ELAM-1 binding activity. Suitable examples of the enzyme include the fucosyltransferase encoded by pCDM7-α(1,3/1,4FT). The plasmid pCDM7-α(1,3/1,4FT) has been deposited in the E. coli strain MC 1069/P3 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 and has been given the accession number 68448. Other examples include an α(1,3)fucosyltransferase capable of constructing Fucα(1,3)GlcNAc linkages on α(2,3)sialylated lactosamine molecules, of the type that is expressed in human myeloid cells (Potvin et al., J. Biol. Chem., vol. 265, 1615-1622 (1990)) or in other species (Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987).

The fucosyltransferase encoded by pCDM7-α(1,3/1,4FT) is preferred and represents the product of the human Lewis blood group locus (Kukowska-Latallo et al., Genes Dev., vol. 4, pp. 1288-1303 (1990)). It exhibits a uniquely broad spectrum of acceptor substrate requirements that encompasses the requirements exhibited by members of two other general classes of α(1,3)fucosyltransferases (Prieels et al., J. Biol. Chem., vol. 256, pp. 10456-10463 (1981); and Palcic et al., Carbohyd. Res., vol. 190, pp. 1-11 (1989)). Expression of this enzyme is thought to be restricted to secretory epithelium, except in Lewis blood group negative individuals who inherit an inability to express this enzyme in any tissue (Watkins, Adv.

Human Genet., vol. 10, pp. 1-116 (1989)). This enzyme is thus probably not responsible for synthesis of the ELAM-1 ligand(s) in the myeloid lineage. The α(1,3)fucosyltransferase encoded by pCDNA1-α(1,3)FT is likewise not responsible since it is representative of a second distinct class of α(1,3)fucosyltransferases (Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987)) that are unable to utilize α(2,3)sialic acid-substituted lactosamine molecules that are the apparent precursors for the ELAM-1 ligand(s). It therefore seems likely that a member of a third class of α(1,3)fucosyltransferases, capable of constructing the Lex and sLex moieties but not the Lea isomers (Potvin et al., J. Biol. Chem., vol. 265, pp. 1615-1622 (1990)), represents the enzyme that determines expression of the ELAM-1 ligand(s) in myeloid cells.

When the starting cells are transfected with pCDM7-α(1,3/1,4FT), suitable starting cells are those that produce substrates which contain carbohydrate moieties corresponding to the sialylated type II precursor shown in Figure 1a:

NeuAcα2→3Galβ1→4GlcNAcα1→R

In a preferred embodiment, the starting cells are immune effector cells whose cell surface oligosaccharide structure does not allow them to be targeted to endothelium expressing ELAM-1. Thus, the present process results in such cells expressing the sLex determinant and being targeted to the areas of inflammation at sites of pathology, where their immune effector functions are therapeutically beneficial. Examples of suitable immune effector cells include lymphokine-activated killer cells (LAK cells) and tumor infiltrating

lymphocytes (TIL cells) in the treatment of advanced cancer, which produce substrates which may be converted to molecules containing the sLex determinant by the enzymes:

(1) a Galβ1->3(4)GlcNAc α(2,3)sialyltransferase (of the type described in Weinstein, J., et al., J. Biol. Chem., 257, 13845-13853 (1982) or the type that operates on O-linked glycoconjugates, as described in Palcic et al., Carbohyd. Res., vol. 190, pp. 1-11 (1989), and

(2) An α(1,3)fucosyltransferase capable of constructing Fucα(1,3)GlcNAc linkages on α(2,3)sialylated lactosamine molecules, of the type described in this application (encoded by pCDM7-α(1,3/1,4)FT, and also described in Palcic et al., Carbohyd. Res., vol. 190, pp. 1-11 (1989) and in Kukowska-Latallo, J.F., et al., Genes Dev., vol. 4, pp. 1288-1303 (1990)), or of the type that is expressed in human myeloid cells (Potvin et al., J. Biol. Chem., vol. 265, 1615-1622 (1990)) or in other species (Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987).

It is to be understood that the starting cells may also be transfected with another heterologous DNA sequence which encodes for a desired polypeptide. Examples of such polypeptides which may be expressed by heterologous DNA sequences include, e.g., EPO, insulin, and interleukin 2.

In another embodiment, the present invention provides a method for targeting a glycoprotein to cells expressing ELAM-1. Thus, a cell which produces a first glycoprotein which can serve as a substrate for an enzyme which will convert the first glycoprotein to a second glycoprotein having a ligand

for ELAM-1 will produce the second glycoprotein after transfection with a suitable enzyme, e.g., the fucosyl transferase encoded by the plasmid pCDM7-α(1,3/1,4FT). Of course, it is to be understood that the cell may produce the first glycoprotein either naturally or as a result of genetic engineering. In another embodiment, the cell may be transfected to produce a second enzyme or a series of enzymes which will convert a first protein or glycoprotein, which is not directly convertable to a glycoprotein having the sialyl Lewis X determinant, to a glycoprotein which can serve as substrate for an enzyme which will convert it to a glycoprotein having the sialyl Lewis X determinant. Thus, the present invention provides cells which produce glycoproteins which are targeted to ELAM-1 and glycoproteins which are targeted to ELAM-1.

Of course the transfection of the starting cells with the DNA sequence encoding for the enzyme responsible for converting the substrate into the ligands for ELAM-1 and any other desired heterologous DNA sequences may be accomplished by any conventional transfection technique. Suitable methods are discussed in Davis et al, Basic Methods in Molecular Biology, Elsevier Publishing Co., NY, NY (1986).

In another embodiment, the carbohydrate ligands for ELAM-1 are linked to recombinant polypeptide antiinflammatory molecules vector by expressing such recombinant proteins, in a form suitable to be expressed with N- and/or O-linked oligosaccharides, in an eukaryotic host that also expresses the enzymes that construct the oligosaccharide ligand(s) for ELAM-1 (i.e., CHO-FT cells, for example).

In another embodiment, the present cells are those which express a substrate on their surface which may be converted to the ligands for ELAM-1 through direct enzymatic action of soluble or immobilized recombinant α(1,3/1,4)FT or its analogous myelod specific enzyme. Thus, the present cells may be produced by a process involving treating a cell with an enzyme which is capable of converting a determinant found on the surface of the cell into a ligand for ELAM-1. It is also possible to treat an oligosaccharide precursor by itself or on another molecule with a recombinant enzyme to form an ELAM-1 ligand.

Thus, in the above-described method, the starting cells need not be transfected.

The present invention also relates to targeting of therapeutics to endothelium expressing ELAM-1 through the construction and linkage of type II structures to liposomes, macromolecular carriers and single molecules. The identification of alpha 1,3 fucosyl transferases as the rate limiting step in the synthesis of ELAM-1 specific ligands, and the demonstration that a cloned alpha 1,3/1,4 fucosyl transferase can generate ligands from type II precursors in a stable transfection system provides both the knowledge and the means to synthesize targeted therapeutics. Thus antiinflammatory agents with systemic toxicity, such as glucocorticoids, methotrexate, cyclosporin and gold salts, could be delivered in high concentration directly to sites of active inflammation.

It is also to be understood that, as in the case of the present compounds of formula (I), the present cells and

liposomes may contain a label suitable for use in an imaging technique in addition to or in replacement of a drug or therapeutic agent. In this way it is possible to target the label to the activated endothelium, and an in vitro imaging technique, such as NMR or radionucleotide scanning, may be used to specifically locate the affected area.

The present results suggest that a leukocyte adhesion deficiency-like phenotype (Anderson and Springer, Ann. Rev. Med., vol. 38, pp. 175-194 (1987)) occurs in individuals with a genetic defect in the ability to express the appropriate α(1,3)fucosyltransferase in the myeloid lineage. In this context, it is noted that many patients with adenocarcinoma, but not healthy individuals, maintain circulating mucin-like molecules containing sialyl Lex Moieties (Kannagi et al., Cancer Res., vol. 46, pp. 2619-2626 (1986)). Such molecules may be shed from malignant tumors in part as a consequence of the "aberrant" expression of the sialyl Lex determinant frequently seen in association with malignant transformation (Fukushi et al., J. Biol. Chem., vol. 259, pp. 4681-4685 (1984); Fukushima et al., Cancer Res., vol. 44, pp. 5279-5285 (1984); and Kannagi et al., Cancer Res., vol. 46, pp. 2619-2626 (1986)). Such molecules may participate in specific interactions with ELAM-1 acting to inhibit normal interactions between leukocytes and the vascular wall, and thereby act to blunt inflammation-induced leukocyte recruitment in these patients. Adenocarcinoma cells have also been shown to exhibit ELAM-1-dependent HUVEC adhesion (Rice and Bevilacqua, Science, vol. 246, pp. 1303-1306 (1989)).

The extensive homology between the CRDs of the LEC-CAM/SELECTIN family raises the possibility that the endogenous

ligands for GMP140/PADGEM and LEC-CAM1 are structurally related to the sLex family of oligosaccharides. The former receptor, in particular, appears to interact with the same spectrum of normal cells and cell lines which adhere to ELAM-1 (Larsen et al. Cell, vol. 59, pp. 305-312 (1989)). Rosen and colleagues have demonstrated that terminally-linked sialic acid is a part of the vascular ligand for $gp90^{MEL14}$ (Yednock and Rosen, Adv. Immunol., vol. 44, pp. 313-378 (1989)). Of potential interest with respect to a fucosylated ligand for this molecule are studies implicating selective inhibition of functional activity with L-fucose, and fucoidan, a heteropolysaccharide rich in fucose-4-sulphate (Stoolman and Rosen, J. Cell Biol., vol. 96, pp. 722-729 (1983); and Stoolman, Tenforde, and Rosen, J. Cell Biol., vol. 99, pp. 1535-1540 (1984)). In recent studies, a direct interaction between two fucose-sulphate containing polysaccharides, fucoidan and the egg-jelly fucan, and purified $gp90^{MEL14}$ has been convincingly demonstrated (Yasuyuki et al., 1990). These synthetic ligands may mimic key structural features or the charge distribution of an endogenous fucosylated sialyl-lactosamine.

In another aspect, the present invention relates to a method of treating a disease mediated by the expression of ELAM-1 by endothelium. Such diseases include, e.g., adult respiratory distress syndrome, vasculitis, and myocardial infarction.

In one embodiment, the treatment of the disease involves administering an effective amount of the compound of the formula (I). When R is not a moiety derived from a drug or therapeutic agent, the compound of formula (I) binds to the

ELAM-1 on the surface of the affected endothelial cells and acts to block the adhesion of the neutrophils. When R is a moiety derived from a drug or represents a drug attached to the sLex determinant via a linking molecule, the compound also acts to specifically deliver the drug to the affected area, in addition retarding the adhesion of the neutrophils to the endothelium.

In another embodiment, the present treatment involves administering cells or liposomes which have been treated according to the present invention so that they carry the ligand(s) for ELAM-1. As in the case of the compounds of formula (I), it is possible to use the present cells and liposomes which carry the carbohydrate ligands on their surfaces as blocking agents. However, it is preferred that the cells and liposomes used in the present treatment contain a drug effective for the treatment of the affected area, in addition to carrying the carbohydrate ligand(s) on their surfaces.

Regardless of whether the present compound, cells or liposomes are being used for the treatment of a disease or the imaging of the affected area, all and any of these may be administered by any conventional means and in any conventional form suitable for placing the compound, cell or liposome in the vasculature. Preferably, the compounds, cells, and liposomes are administered by injection in a form suitable therefore, such as a solution or suspension. Such forms are well known in the art.

The exact dosage of the compound, cells, and liposomes will depend on whether the compound, cells, or liposomes are

being used as a blocking agent or to target drug or label delivery, the identity of the drug or label being delivered, if any, and the size and health of the patient. For any of the above-described purposes or cells, compounds or liposomes, the exact dosage to be administered may be easily determined by one of ordinary skill.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

Examples

Methods:

Cell Culture

Human umbilical vein endothelial cells were isolated and propagated according to the method of Jaffe (Jaffe, in Biology of Endothelial Cells, E.A. Jaffe, Ed., Martinus Nijhoff, The Hague, pp. 1-13 (1984)). Cells were maintained in medium 199 with 20% fetal bovine serum, 100 μg/ml endothelial growth supplement (Collaborative Research), and 100 μg/ml bovine lung heparin (Sigma). Cells were used between passage 2 and 4. Endothelial identity was confirmed by the presence of typical morphology at confluence, by immunofluorescence with antibody to von Willebrand factor, and by uptake of acetylated low density lipoprotein. For adhesion assays, HUVEC were passaged with trypsin/EDTA (Gibco), and plated in 96 well plates (Dynatech, Method I; Falcon, Method II: see below under "HUVEC Adhesion Assays"). Cells were allowed to grow to confluence for at least two days prior to use in adhesion assays. HL-60

lines in spinner culture were maintained between 2.5-15 x 10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. The Chinese hamster ovary (CHO) line Ade-C (Oates and Patterson, Somatic Cell Genet., vol. 3, pp. 561-577 (1977), and Van Keuren et al., Am. J. Human Genet., vol. 38, pp. 793-804 (1986)) was grown in α-modified Eagle's medium supplemented with 10% fetal calf serum. Transfected CHO cells were grown in media supplemented with G418 (Gibco) at 400 μg/ml (active drug). COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Glycosyltransferase Expression Vectors

Previous publications describe the plasmids pCDM7 and pCDM7-αGT (Larsen et al., Proc. Natl. Acad. Sci. USA, vol. 86, pp. 8227-8231 (1989)), pCDM7-α(1,3/1,4)FT (Kukowska-Latallo et al., Genes Dev., vol. 4, pp. 1288-1303 (1990)), and pH3.4 (Rajan et al., J. Biol. Chem., vol. 264, pp. 11158-11167 (1989)). Plasmid pCDNA1-α(1,3)FT contains a 3.6 kb PstI human genomic DNA restriction fragment that encodes an α(1,3)fucosyltransferase (Lowe et al., unpublished). This fragment was isolated from a human lambda phage rescued from a genomic DNA library probed at low stringency with the insert in pCDM7-α(1,3/1,4)FT. The gene segment is cloned in the appropriate transcriptional orientation into the mammalian expression vector pCDNA1 (InVitrogen).

Antibodies

The anti-Le^x antibody anti-SSEA-1 (mouse monoclonal IgM as ascites; Solter and Knowles, Proc. Natl. Acad. Sci. USA, vol. 75, pp. 5565-5569 (1978)) was provided by Dr. D. Solter

(Philadelphia). Anti-sialyl Lex antibody CSLEX1 (mouse monoclonal IgM, HPLC purified; Fukushima et al., Cancer Res. vol. 44, pp. 5279-5285 (1984)) and anti-sialyl Lea antibody CSLEA1 (mouse monoclonal IgG3, ammonium sulfate precipitate; Galton et al., in Antibodies: Protective, Destructive, and Regulatory Role. Ninth Int. Convoc. Immuno., Amherst, NY, pp. 117-125, Karger, Basel (1985); and Chia et al., Cancer Res., vol. 45, pp. 435-437 (1985)) were provided by Dr. P. Terasaki (Los Angeles). Anti-H and anti-Lea[a] antibodies (both mouse monoclonal IgM, antigen affinity purified) were purchased from Chembiomed Ltd. (Edmonton). Anti-ELAM-1 antibody BB11 (Benjamin et al., Biochem. Biophys. Res. Commun., vol. 171, pp. 348-353 (1990)) was the gift of Dr. Roy Lobb (Biogen, Inc., Cambridge, MA). IgG2b, anti-CD11b, anti-CD19, and anti-CD33 antibodies were purchased from Coulter Corp (Hialeah, FLA). Anti-VCAM antibody 4B9 (Carlos and Harlan, Immunol. Rev., vol. 114, pp. 1-24 (1990)) was provided by Dr. John Harlan (University of Washington, Seattle). Anti-ICAM1 antibody 84H10 (Makgoba et al., Nature, vol. 331, pp. 86-88 (1988)) was purchased from AMAC, Inc. Anti-HLA class I antibody W6/32 (Parham et al., J. Immunol., vol. 123, pp. 342-349 (1979)) was purchased from SeraLab, Inc. (U.K).

Transfection, ^{51}Cr-Labeling, and Harvesting of COS-1 cells

COS-1 cells were transfected with various plasmids using the DEAE-dextran procedure (Davis et al., Basic Methods in Molecular Biology, Elsevier Publishing Co., NY, NY, (1986)) as previously described (Larsen et al., Proc. Nat. Acad. Sci. USA, vol. 86, pp. 8227-8231 (1989)). Approximately 72 hours after transfection, cells (approximately 1 x 10^6) were labeled with ^{51}Cr by incubating them for 3-4 hours with 10 μCi/ml of

Na^{51} CrO_4 (NEN). Labeled or unlabeled, transfected COS-1 cells were harvested for HUVEC binding assays, or for flow cytometry analyses, by washing the cell monolayers with calcium, magnesium free PBS (CMF-PBS) containing 2 mM EDTA, and then incubating the washed monolayers with CMF-PBS/2 mM EDTA. Detached cells were washed once by centrifugation through CMF-PBS/2 mM EDTA, and then resuspended in buffers compatible with flow cytometry or binding analyses.

Construction and radiolabeling of stably transfected CHO cell lines

CHO Ade-C cells (Oates and Patterson, Somatic Cell Genet., vol. 3, pp. 561-577 (1977); and Van Keuren et al., Am. J. Hum. Genet., vol. 38, pp. 793-784 (1986)) were transfected (Chen and Okayama, Mol. Cel. Biol., vol. 7, pp. 2745-2752 (1987)) with XhoI-linearized pCDM7-α(1,3/1,4)FT (Kukowska-Latallo et al., Genes Dev., vol. 4, pp. 1288-1303 (1990)), or with XhoI-linearized pCDM7 (Larsen et al., Proc. Natl. Acad. Sci. USA, vol. 86, pp. 8227-8231 (1989)), each co-precipitated in a 10-fold molar excess over EcoRI-linearized pSV2-Neo (Southern and Berg, J. Mol. Appl. Genet., vol. 1, pp. 327-341 (1982)). Approximately 26% of the G418-resistant transfectants generated with pCDM7-α(1,3/1,4)FT stained positively with an anti-Lex antibody (anti-SSEA-1, Solter and Knowles, Proc. Natl. Acad. Sci. USA, vol. 75, pp. 5565-5569 (1978)) . A single, clonal, SSEA-1 positive cell line (CHO-FT) was derived from this population. The G418-resistant transfectants generated with pCDM7 were maintained as an uncloned, pooled population (CHO-V). Cell extracts prepared from CHO-FT contained substantial amounts of α(1,3)fucosyltransferase activity when assayed with the

acceptor N-acetyllactosamine (Kukowska-Latallo et al., Genes Dev., vol. 4, pp. 1288-1303 (1990)), whereas extracts prepared from the parental cell line and from the CHO-V cells contained no detectable fucosyltransferase activity. CHO-FT or CHO-V (6×10^6) were labeled with ^{51}Cr by incubating them for 5-6 hours in 25 μCi/ml of $Na^{51}Cr$. Labelled cells were then harvested using the procedure described above for COS-1 cells.

HUVEC Adhesion Assays

Two methods were used for quantitating heterotypic adhesion to HUVEC monolayers.

Method I

Method I was used for analyses with transfected cells, and is a conventional HUVEC adhesion assay utilizing ^{51}Cr labelled cells incubated at 37°C. HUVEC in 96-well plates were placed in growth media without growth factors, with or without 20 ng/ml TNFα (Genentech), for 4 to 6 hours prior to binding assays. In experiments where inhibitory antibodies were used, antibodies were added in 50 μl PBS containing 100 μg per ml each of Ca and Mg (PBS/Ca/Mg), 0.5% human serum albumin (endotoxin-free, Cutter), and incubated with HUVEC at 4°C for 1 hour. Antibodies were added to a final concentration of 1 μg/ml, except the anti-HLA class I antibody, which ascites used at a 1:1000 dilution. These represent saturating concentrations, as determined by radioimmunoassay. After treating with antibodies, HUVEC monolayers were washed three times with PBS/Ca/Mg. Cells to be tested for adhesion were harvested as detailed above, resuspended in PBS/Ca/Mg, and held at 4°C for the shortest

time possible until used in the assay. Cells (3 x 10^5 neutrophils, 1 x 10^5 COS-1 cells, 3 x 10^5 CHO cells) were added to HUVEC monolayers in 100 μl of PBS/Ca/Mg, and incubated at 37°C. Microscopic titration was used to determine the numbers of cells added of each type (sufficient to just allow cells to form an essentially confluent monolayer overlying the HUVEC monolayer, providing the majority of added cells an opportunity to interact with the underlying endothelium without multilayering). Cells were allowed to adhere for various times (10 to 25 minutes, see Figure legends). Unbound cells were removed by exchanging the wells three times with 150 μl of PBS/Ca/Mg. Each well was then counted in a gamma counter, and the number of bound cells was calculated based upon a previous determination of the number of cpms incorporated per radiolabeled cell.

Method II

Method II maximizes carbohydrate-dependent adhesion by adopting principles used to measure the lectin-dependent adhesion of lymphocytes to frozen sections of lymph nodes (Stoolman et al., Blood, vol. 70, pp. 1842-1850 (1987)). This method was used to determine ELAM-1-dependent adhesion of the HL-60 myeloid cell lines. HUVEC were plated in 96-well tissue culture plates; 24 hours later, the confluent HUVEC monolayers were incubated with 20 ng/ml of TNFα for 4-6 hours prior to initiating binding assays. HL-60 cells were washed in MEM+ (minimal essential medium buffered to pH 7.3 with Tricine, 40 mM, and supplemented with 1 mg/ml bovine serum albumin) immediately prior to fixation. Immediately prior to the binding assay, cells in suspension were fixed in freshly prepared paraformaldehyde (0.5% paraformaldehyde in 0.15 M

cacodylate buffer, pH 7.4; 20 minutes, 4°C), washed extensively, and resuspended at 10^6 cells/ml in MEM+. In some experiments, the TNFα-treated HUVEC monolayers were preincubated at 7-10°C with 50 μl of either IgG2b or BB11 monoclonal antibodies at a concentration of 20 μ/ml. Fixed cells (10^5 cells in 50 μl) were generally added directly to the plates after 15-30 minutes of preincubation (washing the HUVEC monolayers free of unbound antibody prior to instituting the binding assays had no effect on the level of inhibition, data not shown). Added cells were allowed to settle and interact with the HUVEC for 45 minutes at 7-10°C prior to quantitation of adhesion.

Unattached cells were removed by aspirating the cell suspensions and gently washing the monolayers three times with 200 μl of MEM+ at room temperature. The wash solution was applied with an 8-channel multipipettor to the upper wall of the wells while maintaining the plates at a 30° angle. The solutions were then drawn off the lower wall without contacting the monolayer. The number of cells recovered from each well was determined by counting the pooled washes on a Coulter ZBI cell counter. The number of bound cells represents the difference between the number of cells applied to each well and the number recovered from the well.

Flow Cytometry

Transfected COS-1 cells, or CHO transfectants, were subjected to flow cytometry analysis with mouse monoclonal IgM antibodies directed against carbohydrate epitopes, using procedures described previously (Ernst et al., J. Biol. Chem., vol. 264, pp. 3436-3447 (1989); and Kukowska-Latallo et al.,

Genes Dev., vol. 4, pp. 1288-1303 (1990)). Cells were stained with saturating amounts of anti-SSEA-1 monoclonal antibody (1:1000 dilution of ascites), anti-Lewis a monoclonal antibody (10 μg/ml), anti-H antibody (10 μg/ml), or anti-CSLEX1 (10 μg/ml). Cells were then stained with fluorescein-conjugated goat anti-mouse IgM (Sigma; 40 μg/ml) and subjected to analysis by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA), as described previously (Ernst et al., J. Biol. Chem., vol. 264, pp. 3436-3447 (1989); and Larsen et al., Proc. Natl. Acad. Sci. USA, vol. 86, pp. 8227-8231 (1989)). Cell staining was measured in arbitrary fluorescent intensity units, and was displayed on a four decade log scale. Analyses with the IgG3 class mouse anti-CSLEA1 antibody (10.8 μg/ml), or the pooled mouse IgG control antibody (MsIg, Coulter, 10 μg/ml) were performed in an identical manner, except that a FITC-conjugated goat antimouse IgG antibody (Sigma, 40 μg/ml) was used.

Flow cytometry-based indirect immunofluorescence analyses of HL-60 cells, were conducted in microtiter plates. Both primary and secondary antibodies were used at saturating concentrations. Antigen specific mouse anti-human monoclonal antibodies (50 μL) were combined with cells (2.5×10^5 in 50 μL) and incubated at 4°C for 30 minutes in PBS+ (phosphate buffered saline supplemented with 1% fetal bovine serum and 0.05% Na azide). Cells were washed twice in PBS+ (200 μL), resuspended and combined with 50 μL of FITC-labeled goat anti-mouse IgG (Fab' 2 fragments, Cappel Laboratories, West Chester, PA) for an additional 30 minutes at 4°C. After three washes in PBS+, pellets were resuspended and combined with 200 μL of 1-2% paraformaldehyde prior to cytometric analysis. Fluorescence quantitation was performed on a Coulter Epics V

Flow Cytometer equipped with a three-decade scale.

Results

ELAM-1 Dependent Cell Adhesion Correlates with sLex Expression in Variants of the HL-60 Cell Line

The ELAM-1 receptor mediates the adhesion of neutrophils, monocytes, and the related cell lines HL-60 and U937 to cytokine-stimulated human umbilical vein endothelial cells (HUVEC) (Bevilacqua et al., Proc. Natl. Acad. Sci. USA, vol. 84, pp. 9238-9242 (1987); and Bevilacqua et al., Science, vol. 243, pp. 1160-1165 (1989)). The surfaces of these leukocytic cells are unusually rich in fucosylated derivatives of neutral and α(2,3)sialylated polylactosamine ([Galβ1,4GlcNAc)$_n$]) moieties (Fukuda et al. J. Biol. Chem., vol. 259, pp. 10925-10935 (1984); Fukuda et al., J. Biol. Chem., vol. 260, pp. 1067-1082 (1985); and Spooncer et al., J. Biol. Chem., vol. 259, pp. 4792-4801 (1984)). The structurally and biosynthetically related members of this group include the sialyl Lewis X tetrasaccharide (NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAc, sLex; Fukushima et al., Cancer Res., vol. 44, pp. 5279-5285 (1984); Figure 1a), its non-sialylated trisaccharide analogue Lewis X (Galβ1→4(Fucα1→3)GlcNAc, Lex or SSEA-1; Gooi et al., Nature, vol. 292, pp. 156-158 (1981); Figure 1A), and related neutral and α(2,3)sialic acid-substituted structures containing single (VIM-2, Macher et al., J. Biol. Chem., vol. 263, pp. 10186-10191 (1988)) or multiple (FH4 and FH5, Fukushi et al., J. Biol. Chem., vol. 259, pp. 4681-4685 (1984); FH6, Fukushi et al., Cancer Res., vol. 45, pp. 3711-3717 (1985)) internal α(1,3)-linked fucose residues. By contrast, these

oligosaccharide determinants are generally not detected on circulating cells which lack the ligand(s) for ELAM-1 (Fox et al., Cancer Res., vol. 43, pp. 669-678 (1983); Fukushima et al., Cancer Res., vol. 44, pp. 5279-5285 (1984); and Fukushi et al., Cancer Res., vol. 45, 3711-3717 (1985). These observations prompted us to measure the expression of sLex and Lex epitomes on two variants of the HL-60 line showing markedly different levels of ELAM-1-dependent adhesion to TNFα-treated HUVEC.

Figure 2 depicts a series of experiments with two spontaneously arising variants of the HL-60 line. Both cell lines expressed the myeloid differentiation antigens CD11b, CD19 and CD33 (data not shown). Using an assay that maximizes carbohydrate-dependent cell adhesion (Stoolman et al., Blood, vol. 70, 1842-1850 (1987) and Experimental Procedures), HL-60 line B cells bound avidly to TNFα-treated HUVEC, with 55% of the added cells attached in the presence of the control antibody IgG2b. This adhesion was largely ELAM-1-dependent since only 11% of the cells adhered in the presence of the anti-ELAM-1 antibody BB11 (Benjamin et al., Biochem. Biophys. Res. Commun., vol. 171, pp. 348-353 (1990)). By contrast, the line designated HL-60 A exhibited a lower absolute level of adhesion (mean of 16%) which was not significantly reduced by treatment of the HUVEC with BB11 (14%). Thus HL-60 A showed virtually no ELAM-1-dependent adhesion under conditions that optimized detection of this interaction on HUVEC monolayers.

Flow cytometer analysis revealed markedly different levels of the sLex and Lex determinants at the cell surface of HL-60 A and HL-60 B (Figure 2b). Both cell lines expressed the Lex epitope as has been previously described for the HL-60

cell line (Symington et al., J. Immun., vol. 134, pp. 2498-2506 (1985)). By contrast, the sLex epitope was detected on the adhesion competent HL-60 line B cells exclusively. The resistance of the ELAM-1 ligand(s) to paraformaldehyde fixation and the persistence of its functional activity at reduced temperature mirror the behavior of the endogenous, sialylated ligand for LEC-CAM1 (Stoolman, Cell, vol. 56, pp. 907-910 (1989); and Yednock and Rosen, Adv. Immunol., vol. 44, pp. 313-378 (1989)), and thus are consistent with the hypothesis that the CRD of ELAM-1 interacts with an oligosaccharide on the surface of HL-60 cells. Moreover, the coordinate loss of both ELAM-1-dependent adhesion and the sLex determinant suggested that the ligand(s) belongs to the family of sialylated, fucosylated polylactosaminoglycans expressed at high levels on myeloid cells.

ELAM-1 Dependent Cell Adhesion of COS-1 Cells Determined by Transfection of a Cloned Fucosyltransferase cDNA

The profound differences in sLex expression observed in the HL-60 variants may have resulted from differences in the activities of specific fucosyltransferases (Figure 1a), by analogy to the Chinese hamster ovary (CHO) glycosylation mutants Lec11 and Lec12 (Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987)). The LEC11 mutant exhibits de novo expression of a specific α(1,3)fucosyltransferase, termed Fuc-TI, that determines the synthesis of surface-localized neutral and α(2,3)sialylated polylactosaminoglycans substituted with α(1,3)-linked fucose residues (Figure 1a). By contrast, the Lec12 mutant expresses a distinct α(1,3)fucosyltransferase, Fuc-TII, that determines surface display of neutral, α(1,3)-fucosylated polylactosaminoglycans,

but not the α(2,3)sialylated analogues (Figure 1a). The penultimate step in the biosynthesis of these structures, which include the sialyl Lex moiety, is thought to be catalyzed by a widely distributed sialyltransferase that attaches sialic acid in an α(2,3) linkage to terminal galactose residues in the polylactosamine substrate (Weinstein et al., J. Biol. Chem., vol. 257, pp. 13845-13853 (1982)). These sialylated molecules can then serve as acceptors for some (Holmes et al., J. Biol. Chem., vol. 261, pp. 3737-3743 (1986); and Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987)), but not all (Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987)) α(1,3)fucosyltransferases. These enzymes may add fucose residues in an α(1,3)-linkage to the terminal GlcNAc residue, thus forming the sLex structure, and/or to one or more GlcNAc residues within internal lactosamine units (Holmes et al., J. Biol. Chem., vol. 261, pp. 3737-3743 (1986); and Howard et al., J. Biol. Chem., vol. 262, pp. 16830-16837 (1987)). Likewise, α(1,3)fucosyltransferases can generate the Lex moiety and its polyfucosylated analogues from neutral polylactosamine precursors. These neutral fucosylated molecules are not, however, substrates for any known α(2,3)sialyltransferases. These considerations suggest a critical, regulatory role for expression of specific α(1,3)fucosyltransferases in the biosynthesis of the family of sialylated, fucosylated lactosaminoglycans that represent putative ELAM-1 ligands.

To investigate this possibility, and to test the roles of these molecules in ELAM-1-dependent cell adhesion, their de novo surface expression on mammalian host cells was effected, via modification of their glycosylation phenotypes with

transfected α(1,3)fucosyltransferase cDNAs or gene segments (pCDM7-α(1,3/1,4)FT, Kukowska-Latallo et al. Genes Dev., vol. 4, pp. 1288-1303 (1990); and pCDNA1-α(1,3)FT, Lowe et al., unpublished). Non-myeloid hosts were chosen for these experiments in order to isolate the role of the oligosaccharide molecules in ELAM-1-dependent adhesion from other myeloid-specific molecules known to participate in leukocyte-endothelial cell interactions (Springer, Nature, vol. 346, pp. 425-434 (1990)). COS-1 cells were used for the first of these experiments since these cells do not express detectable α(1,3)fucosyltransferase activity nor cell surface oligosaccharides that contain the cognate α(1,3)fucose linkages (Kukowska-Latallo et al., Genes Dev., vol. 4, pp. 1288-1303 (1990)). Moreover, COS-1 cells do not exhibit ELAM-1-dependent adhesive properties. They do, however, express the oligosaccharide substrates necessary for α(1,3)fucosyltransferase-dependent biosynthesis of the family of oligosaccharides represented by the Lex and sialyl Lex molecules (Fukuda et al., J. Biol. Chem., vol. 263, pp. 5314-5318 (1988); and Kukowska-Latallo et al., Genes Dev., vol. 4, pp. 1288-1303 (1990)).

As shown in Figure 3a, 26-31% of the population of COS-1 cells transfected with a plasmid encoding an α(1,3/1,4)fucosyltransferase (pCDM7-α(1,3/1,4)FT, Kukowska-Latallo el al. Genes Dev., vol. 4, pp. 1288-1303 (1990)) express sLex and Lex molecules (Figure 3a). As expected from the properties of the enzyme encoded by pCDM7-α(1,3/1,4)FT (Prieels et al., J. Biol. Chem., vol. 256, pp. 10456-10463 (1981); Palcic et al., Carbohyd. Res., vol. 190, pp. 1-11 (1989); and Kukowska-Latallo et al., Genes Dev., vol. 4, pp. 1288-1303 (1990)), the sLea and Lea determinants

are also expressed (Figures 1b and 3a). By contrast, COS-1 cells transfected with pCDNA1-α(1,3)FT, which encodes a distinct α(1,3)fucosyltransferase (Lowe et al., unpublished), express new Lex determinants exclusively (Figure 3a). Cells transfected with the control vectors (pCDM7 and pCDNA1), as well as with vectors encoding two other mammalian glycosyltransferases (pCDM7-αGT, Larsen et al., Proc. Natl. Acad. Sci. USA, vol. 86, pp. 8227-8231 (1989); and pH3.4, Rajan et al., J. Biol. Chem., vol. 264, pp. 11158-11167 (1989)) remained negative for all four surface carbohydrate determinants (Figure 3a and data not shown). Using ^{51}Cr-labeled cells, only pCDM7-α(1,3/1,4)FT transfectants, expressing the four new fucosylated oligosaccharide determinants, exhibited adhesion to TNFα-treated HUVEC (Figure 3b). This binding was substantially inhibited with the anti-ELAM-1 antibody BB11, but not with its isotype control antibody IgG2b, nor with antibodies that detect other polypeptides expressed on TNFα-treated HUVEC (VCAM1, ICAM1, and HLA class I determinants, Figure 3c).

In these experiments, approximately 7% of the pCDM7-α(1,3/1,4)FT transfectants adhered to TNFα-treated endothelium, whereas 35% of normal neutrophils adhered under the same conditions (Figure 3b, right). This apparent difference in relative adhesion "activity" can be accounted for by the fact that only a minority (26-31%) of the radiolabeled COS-1 cells actually express new cell surface oligosaccharide determinants, and by the fact that ELAM-1 interactions represent only one of several mechanisms responsible for adhesion of neutrophils to cytokine-stimulated endothelium (Luscinska et al., J. Immunol., vol. 142, pp. 2257-2263 (1989)). Indeed, pretreatment of the TNFα-activated HUVEC in

this experiment incompletely blocked neutrophil adhesion, reducing it to approximately 17% (data not shown). These considerations indicate that ELAM-1-dependent adhesion of the two cell types is of a substantially similar magnitude.

The neutral and sialylated Lea isomers (Figure 1b) displayed by the pCDM7-α(1,3/1,4)FT transfected, adhesion-competent COS-1 cells are generally absent from blood cells of the myeloid lineage (Fukuda et al., J. Biol. Chem., vol. 259, pp. 10925-10935 (1984); Fukuda et al., J. Biol. Chem., vol. 260, pp. 1067-1082 (1985); Spooncer et al., J. Biol. Chem., vol. 259, pp. 4792-4801 (1984); and Dunstan, Br. J. Haemat., vol. 62, pp. 301-309 (1986)) and thus would not normally participate in leukocyte-ELAM-1 adhesive interactions. It is therefore concluded that the ELAM-1-dependent adherence of cells transfected with pCDM7-α(1,3/1,4)FT is mediated by the surface-localized sialyl Lex tetrasaccharide molecule, and/or its alternately and poly α(1,3)fucosylated analogues, but not by the neutral family of molecules represented by the Lex determinant.

Expression of a Cloned Fucosyltransferase cDNA in Stably-transfected CHO Cells Determines ELAM-1 Dependent Cell Adhesion and sLex Expression

To isolate and further confirm the contribution of the sialylated Lex-type oligosaccharide molecules to ELAM-1-dependent binding, we generated a transfected mammalian cell line (CHO-FT) that expresses surface-localized sLex and Lex determinants, but not the Lea or sLea determinants. The parental CHO cell line Ade$^-$ C (Oates et al., Somatic Cell Genet., vol. 3, pp. 561-577 (1977); and Van Keuren el al., Am.

J. Hum. Genet., vol. 38, pp. 793-804 (1986)) chosen for these experiments expresses no detectable α(1,3)fucosyltransferase activity (vide supra) nor the corresponding Lex and sialyl Lex molecules (Smith et al., J. Biol. Chem., vol. 265, pp. 6225-6234 (1990)). It does, however, express polylactosaminoglycan precursors for these molecules, but not for the Lea precursors (Smith et al., J. Biol. Chem., vol. 265, pp. 6225-6234 (1990)). Expression of stably-transfected pCDM7-α(1,3/1,4)FT in this host determines surface display of substantial amounts of the sLex determinant, and to a lesser extent the Lex determinant, but not the Lea isomers (Figure 4a).

Each CHO transfectant line was assessed for adhesion to untreated and TNFα-treated HUVEC (Figures 4b and 4c). Cells were allowed to adhere for between 5 and 25 minutes, before washing off unbound cells. CHO-FT cells exhibited marked adhesion to TNFα-treated HUVEC, but not to untreated endothelium (Figure 4b). Adhesion of CHO-FT cells was well-established within 5 minutes, peaked at 10 minutes, and was resistant to repeated and vigorous washing (vide supra). By contrast, control transfectants (CHO-V), that do not express significant amounts of the Lex or sialyl Lex molecules (Figure 4a), did not exhibit meaningful binding to either TNFα-treated or untreated HUVEC (Figure 4b) at early time points, and minimal, non-specific adhesion at later ones. This adhesion was ELAM-1-dependent since it was inhibited by pretreatment of the HUVEC with the BB11 anti-ELAM-1 antibody, but not by the control antibody IgG2b, nor with antibodies directed against other adhesion receptors (VCAM1 and ICAM1), or to HLA class I determinants (Figure 4c and data not shown). The very rapid onset of the ELAM-1-dependent adhesion to vascular endothelium exhibited by the CHO-FT cells is physiologically consistent

with the speed of the analogous interaction between neutrophils and ELAM-1, that arrests these latter cells as they pass over activated endothelium.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

Claims:

1. A compound of formula (I):

NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcα1→R (I)

wherein R is OH, a protected hydroxy group, a C_{1-12} alkyl group, $-O-(CH_2)_n-CO_2R''$ (wherein R" is C_{1-4} alkyl and n is 2 to 12), $-O-(CH_2)_nCONHNH_2$ (n is 2 to 12), $-O-(CH_2)_nCON_3$ (n is 2 to 12), a lipid, a glycolipid, a glycoconjugate of a serine/threonine-linked oligosaccharide or a protein or a polypeptide, a glycoconjugate of a free oligosaccharide derived by chemical or enzymatic hydrolysis of serine/threonine-linkage to a protein or polypeptide, a glycoconjugate of an asparagine-linked oligosaccharide on a protein or polypeptide, or a glycoconjugate of a free oligosaccharide derived by enzymatic or chemical hydrolysis from an asparagine linkage to a protein or polypeptide, a moiety derived from a drug, a moiety derived from a fluorescent or chemiluminescent label, or a group of the formula -X-R', wherein X is a bivalent linking group and R' is a moiety derived from a drug or a moiety derived from a fluorescent or chemiluminescent label.

2. A method for treating a disease mediated by the elaboration of ELAM-1 on endothelial cells, comprising administering a therapeutically effective amount of a compound of Claim 1, to a patient in need thereof.

3. The method of Claim 2, wherein said disease is selected from the group consisting of adult respiratory

distress syndrome, vasculitis, and myocardial infarction.

4. A transfected cell, which contains a heterologous sequence of DNA which encodes an enzyme which exhibits the activity of converting a substrate produced by the cell to the sialyl Lewis X determinant.

5. The cell of Claim 4, wherein said enzyme is the fucosyltransferase encoded by pCDM7-α(1,3/1,4FT).

6. A process for preparing a transfected cell, comprising:

(i) transfecting a cell with a heterologous sequence of DNA encoding an enzyme having the activity of converting a substrate produced by said cell to the sialyl Lewis X determinant.

7. The process of Claim 6, wherein said enzyme is the fucosyltransferase encoded by pCDM7-α(1,3/1,4FT).

8. A cell, prepared by a process, comprising:

(i) treating a cell with an enzyme which has the activity of converting a compound on the surface of said cell into a compound which contains the sialyl Lewis X determinant.

9. The cell of Claim 8, wherein said enzyme is the fucosyltransferase encoded by pCDM7-α(1,3/1,4FT).

10. A process for preparing an oligosaccharide, comprising:

(i) treating a cell with an enzyme which has the activity of converting a compound on the surface of said cell into a compound which contains the sialyl Lewis X determinant.

11. The process of Claim 10, wherein said enzyme is the fucosyltransferase encoded by pCDM7-α(1,3/1,4FT).

12. In a method for imaging an area of inflammation of the endothelium by NMR or radionucleotide scanning, the improvement being the use of (i) a compound of the formula (I)

NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcα1→R (I)

wherein R is a moiety derived from a label suitable for said imaging or a group of the formula -X-R' wherein X represents a bivalent linking group and R' represents a moiety derived from a label suitable for said imaging; (ii) a cell which expresses the sialyl Lewis X determinant and contains a label suitable for said imaging or; (iii) a liposome which carries on its surface the sialyl Lewis X determinant and comprises a label suitable for said imaging, to target said label to said area of inflammation.

13. A liposome, wherein the sialyl Lewis X determinant is present on the surface of said liposome.

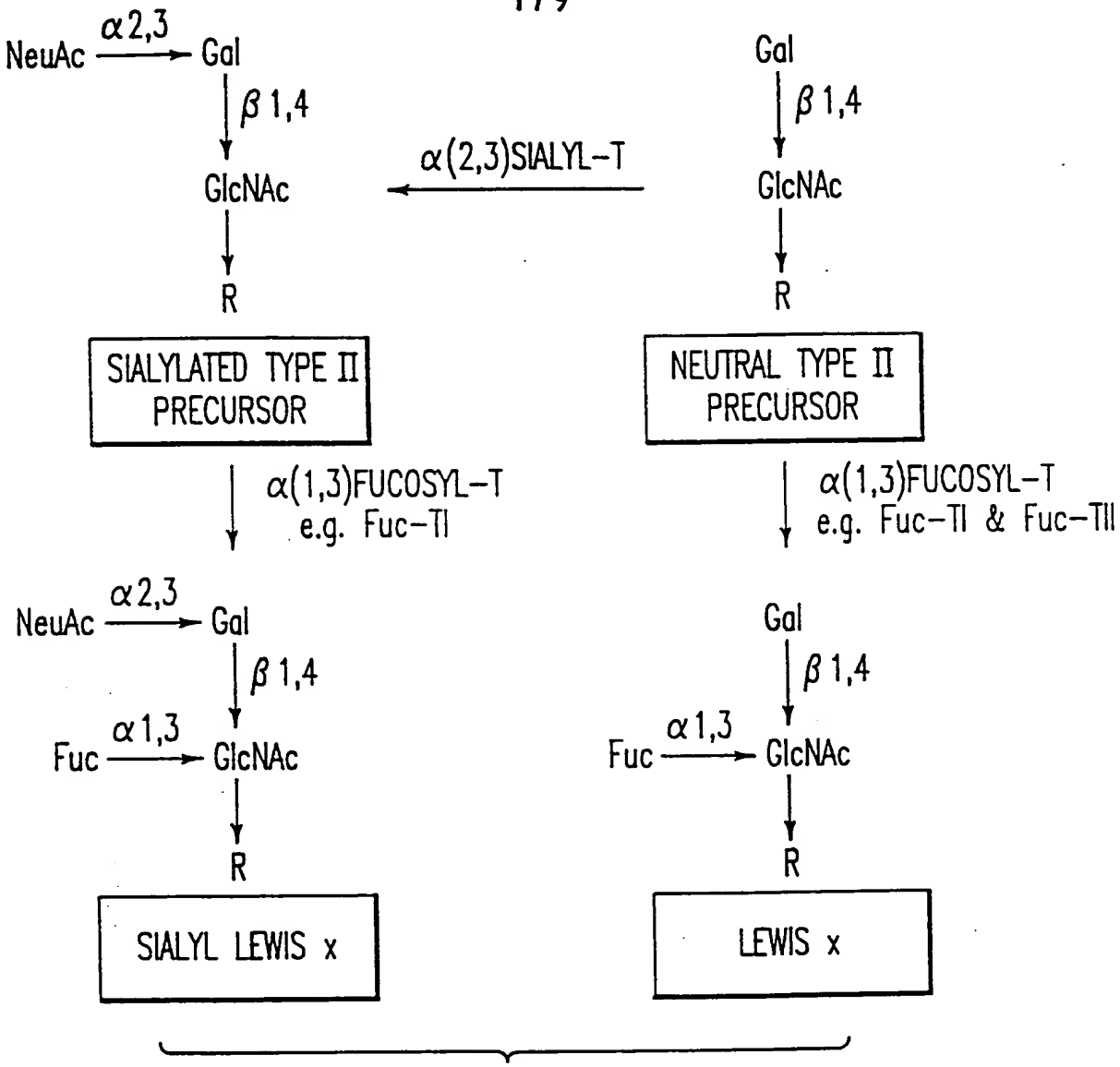
NeuAc α2,3 Gal β 1,4 GlcNAc R
α(2,3)SIALYL-T
Gal β 1,4 GlcNAc R
SIALYLATED TYPE II PRECURSOR
NEUTRAL TYPE II PRECURSOR
α(1,3)FUCOSYL-T e.g. Fuc-TI
α(1,3)FUCOSYL-T e.g. Fuc-TI & Fuc-TII
NeuAc α2,3 Gal β 1,4 Fuc α1,3 GlcNAc R
Gal β 1,4 Fuc α1,3 GlcNAc R
SIALYL LEWIS x
LEWIS x

FIG. 1A

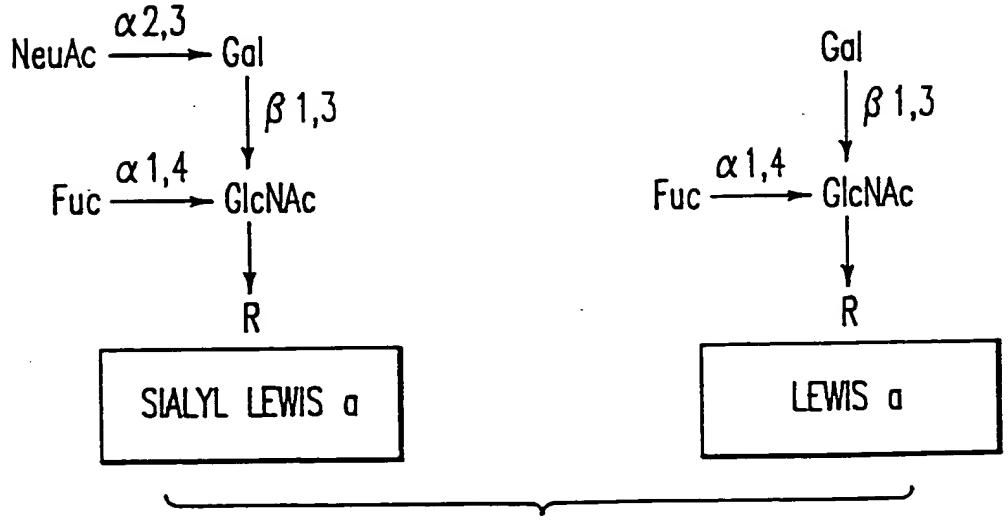
NeuAc α2,3 Gal β 1,3 Fuc α1,4 GlcNAc R
Gal β 1,3 Fuc α1,4 GlcNAc R
SIALYL LEWIS a
LEWIS a

FIG. 1B

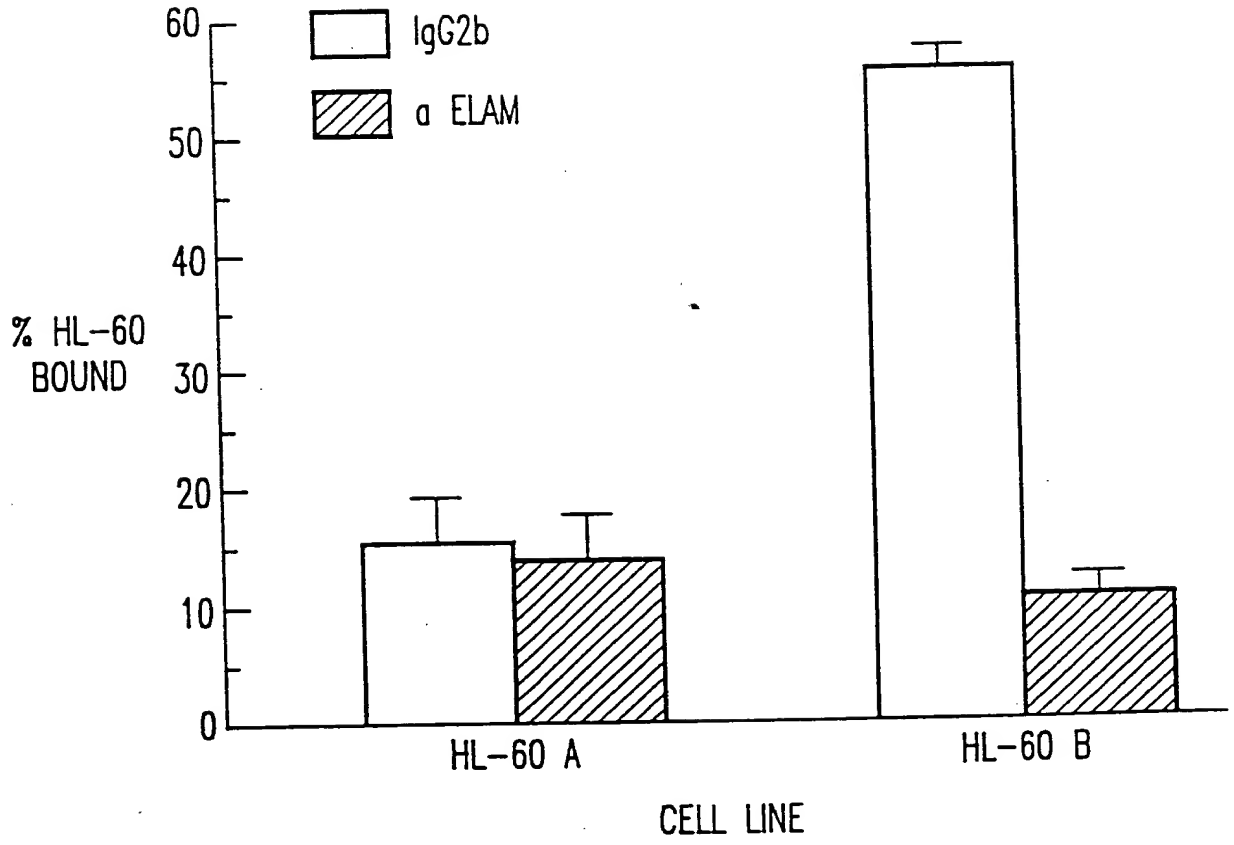
60
50
40
30
20
10
0
% HL-60 BOUND
IgG2b
α ELAM
HL-60 A
HL-60 B
CELL LINE

FIG. 2A

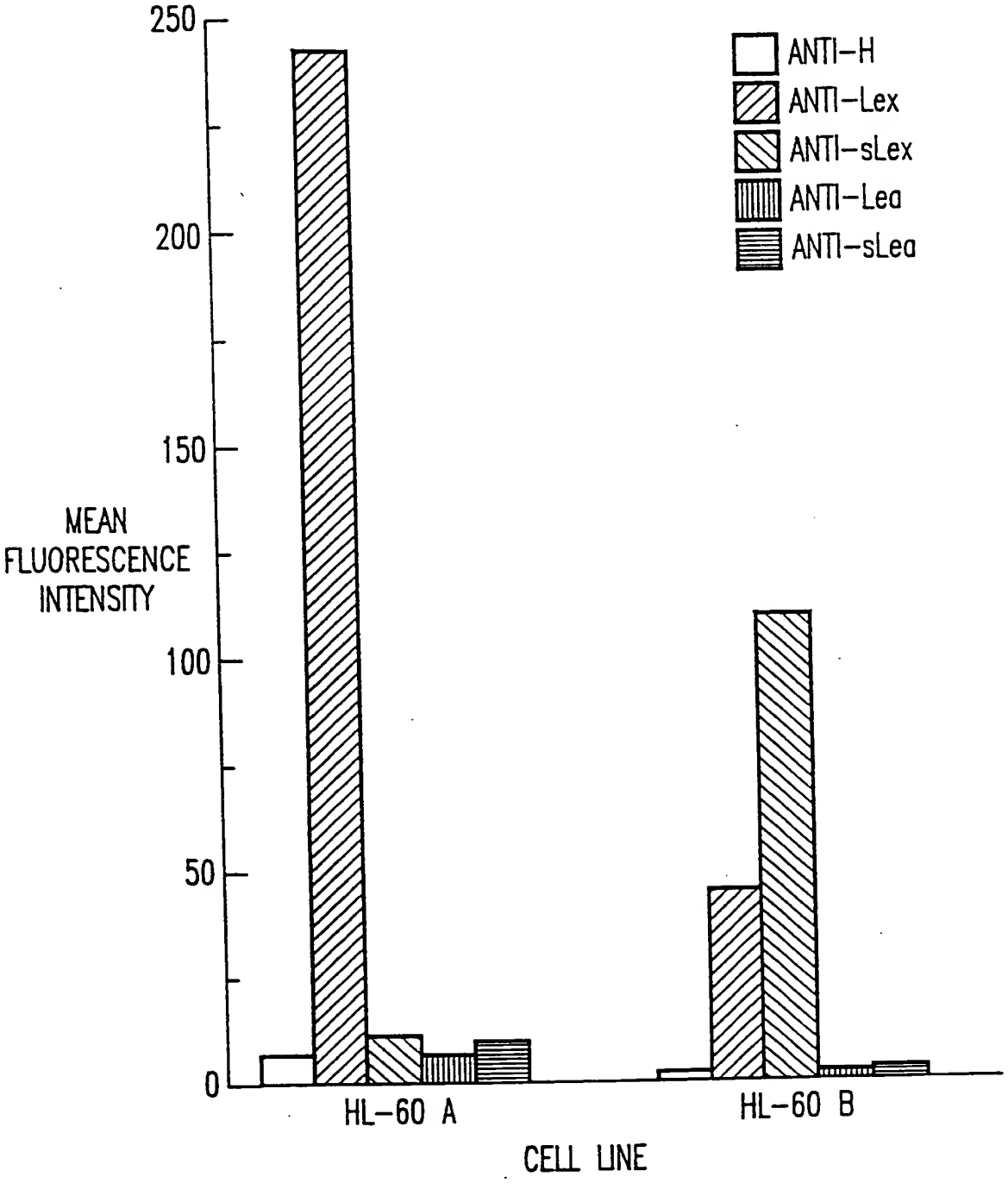
250
200
150
100
50
0
MEAN FLUORESCENCE INTENSITY
ANTI-H
ANTI-Lex
ANTI-sLex
ANTI-Lea
ANTI-sLea
HL-60 A
HL-60 B
CELL LINE

FIG. 2B

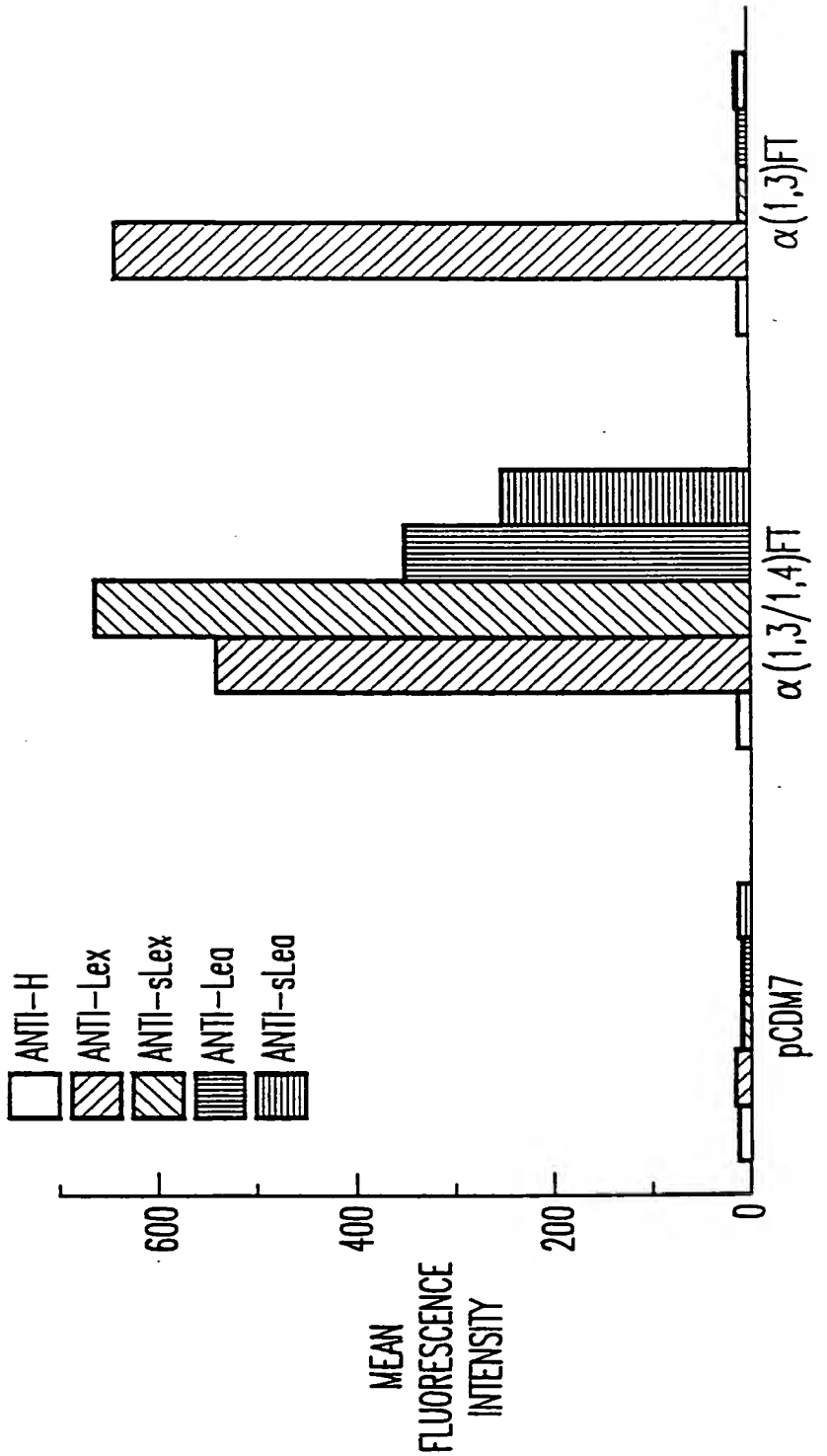
ANTI-H
ANTI-Lex
ANTI-sLex
ANTI-Lea
ANTI-sLea
MEAN FLUORESCENCE INTENSITY
0
200
400
600
pCDM7
α(1,3/1,4)FT
α(1,3)FT
VECTOR

FIG. 3A

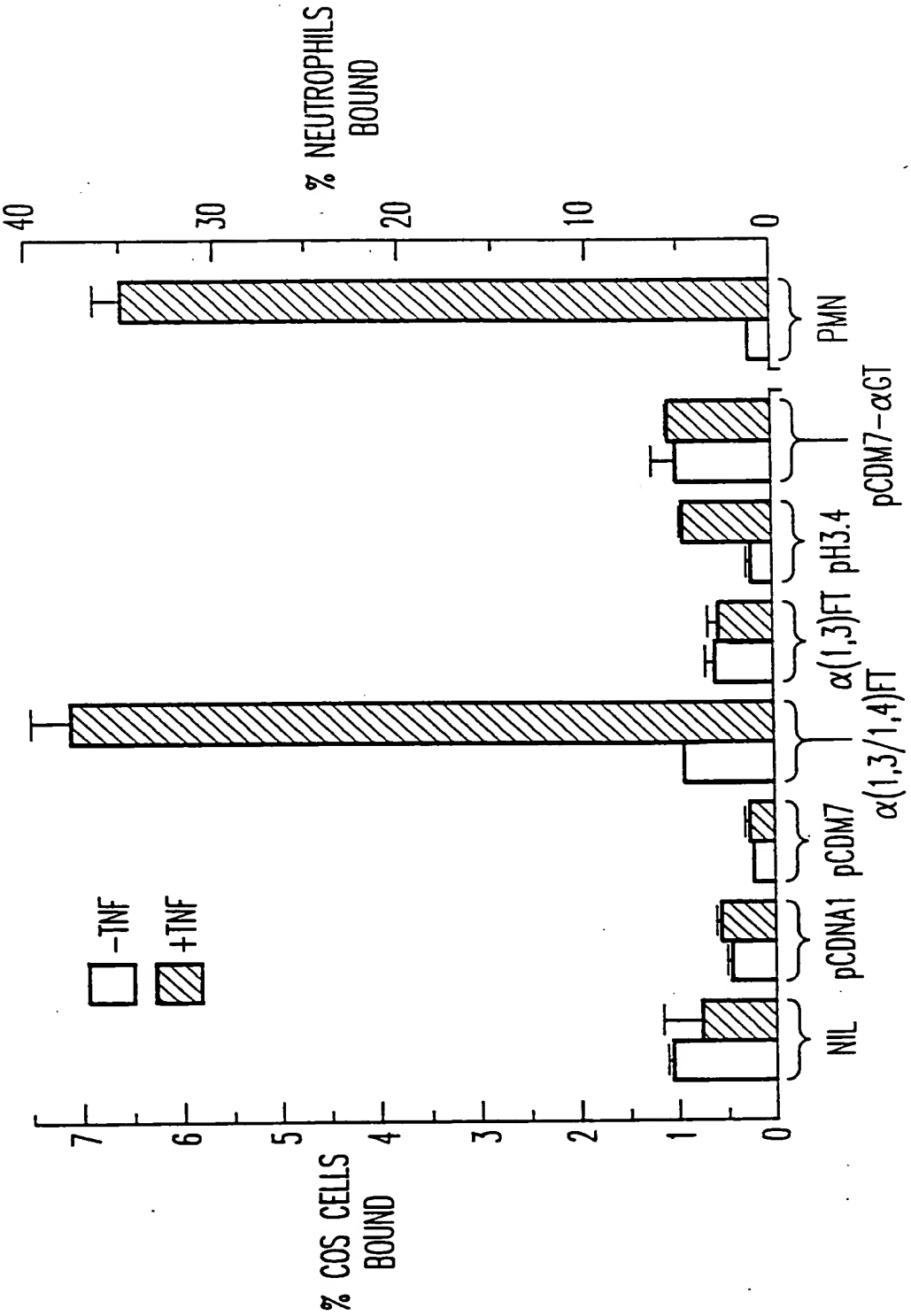
% NEUTROPHILS BOUND
40
30
20
10
0
PMN
pCDM7-αGT
α(1,3)FT pH3.4
α(1,3/1,4)FT
pCDM7
pCDNA1
NIL
-TNF
+TNF
% COS CELLS BOUND
7
6
5
4
3
2
1
0

FIG. 3B

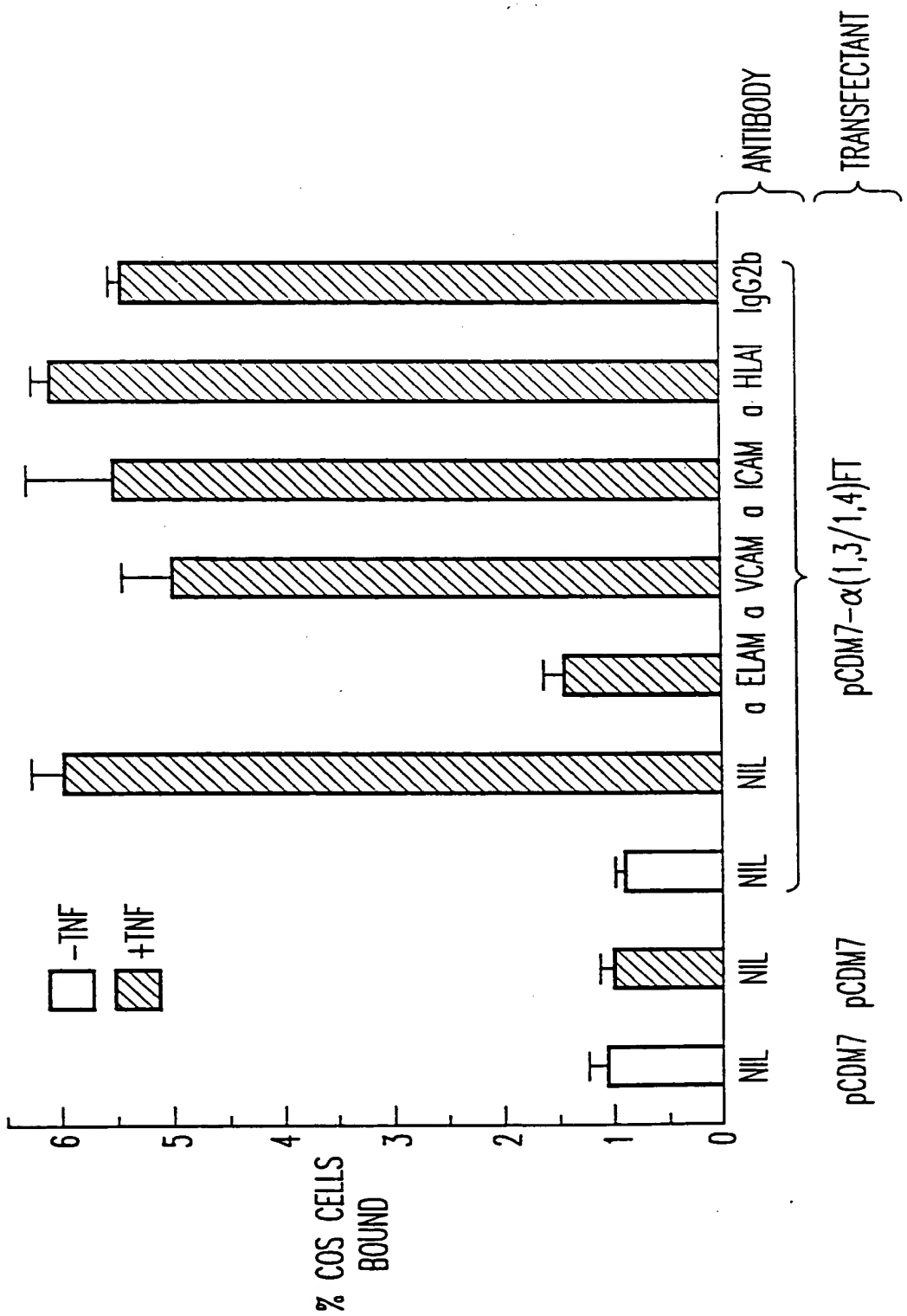
% COS CELLS BOUND
0
1
2
3
4
5
6
-TNF
+TNF
NIL
NIL
NIL
NIL
a ELAM
a VCAM
a ICAM
a HLAI
IgG2b
pCDM7
pCDM7
pCDM7-α(1,3/1,4)FT
TRANSFECTANT
ANTIBODY

FIG. 3C

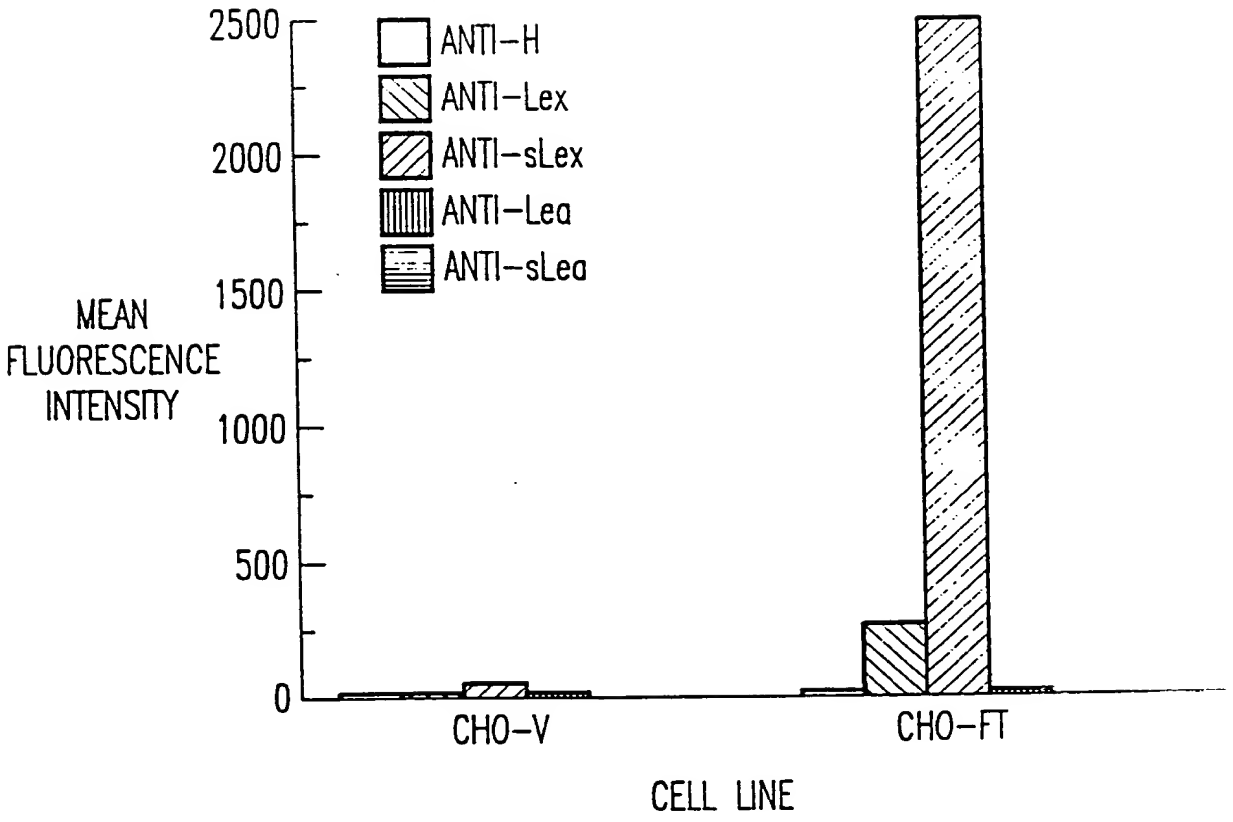
2500
2000
1500
1000
500
0
MEAN
FLUORESCENCE
INTENSITY
ANTI-H
ANTI-Lex
ANTI-sLex
ANTI-Lea
ANTI-sLea
CHO-V
CHO-FT
CELL LINE

FIG. 4A

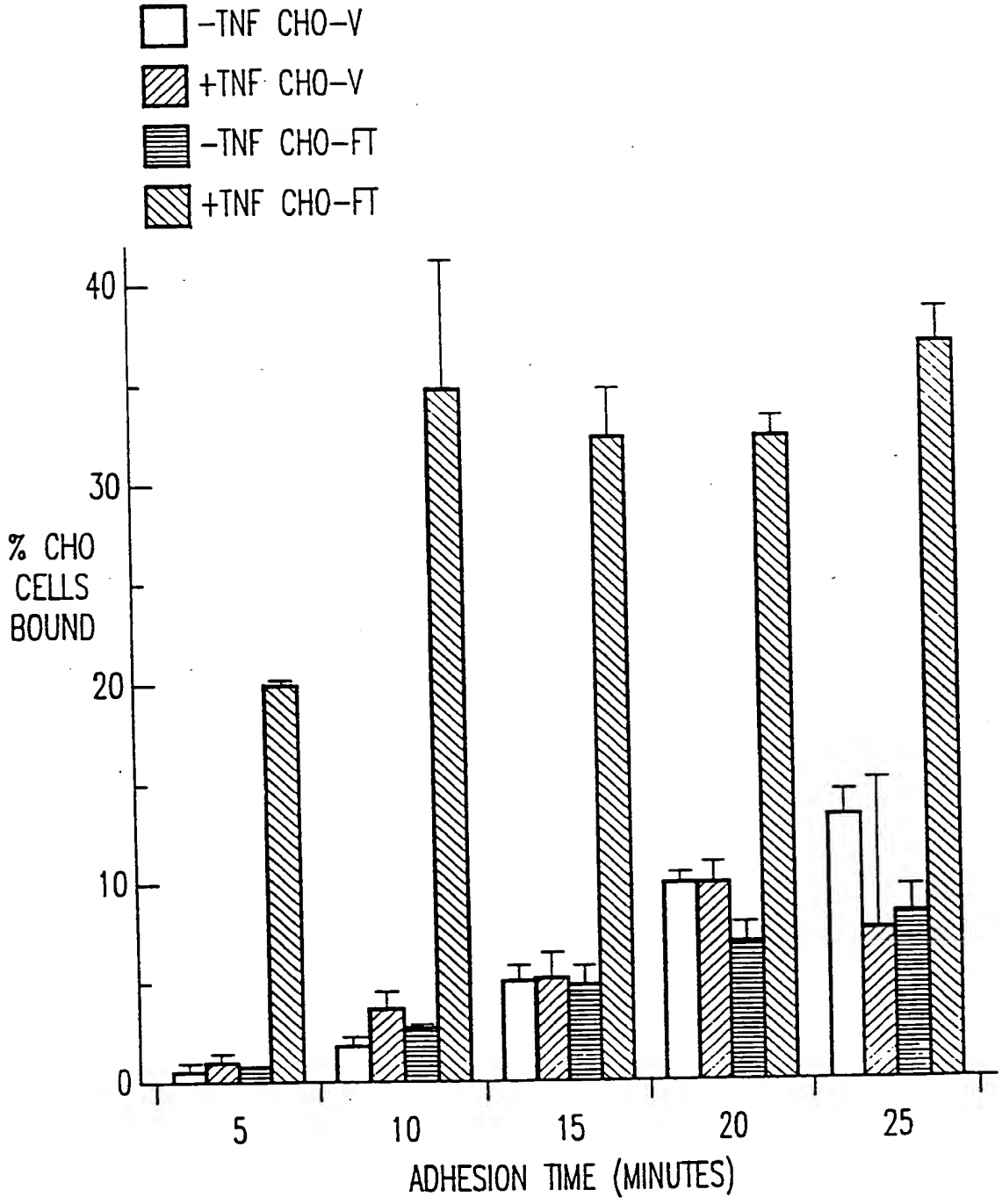
-TNF CHO-V
+TNF CHO-V
-TNF CHO-FT
+TNF CHO-FT
% CHO CELLS BOUND
0
10
20
30
40
5
10
15
20
25
ADHESION TIME (MINUTES)

FIG. 4B

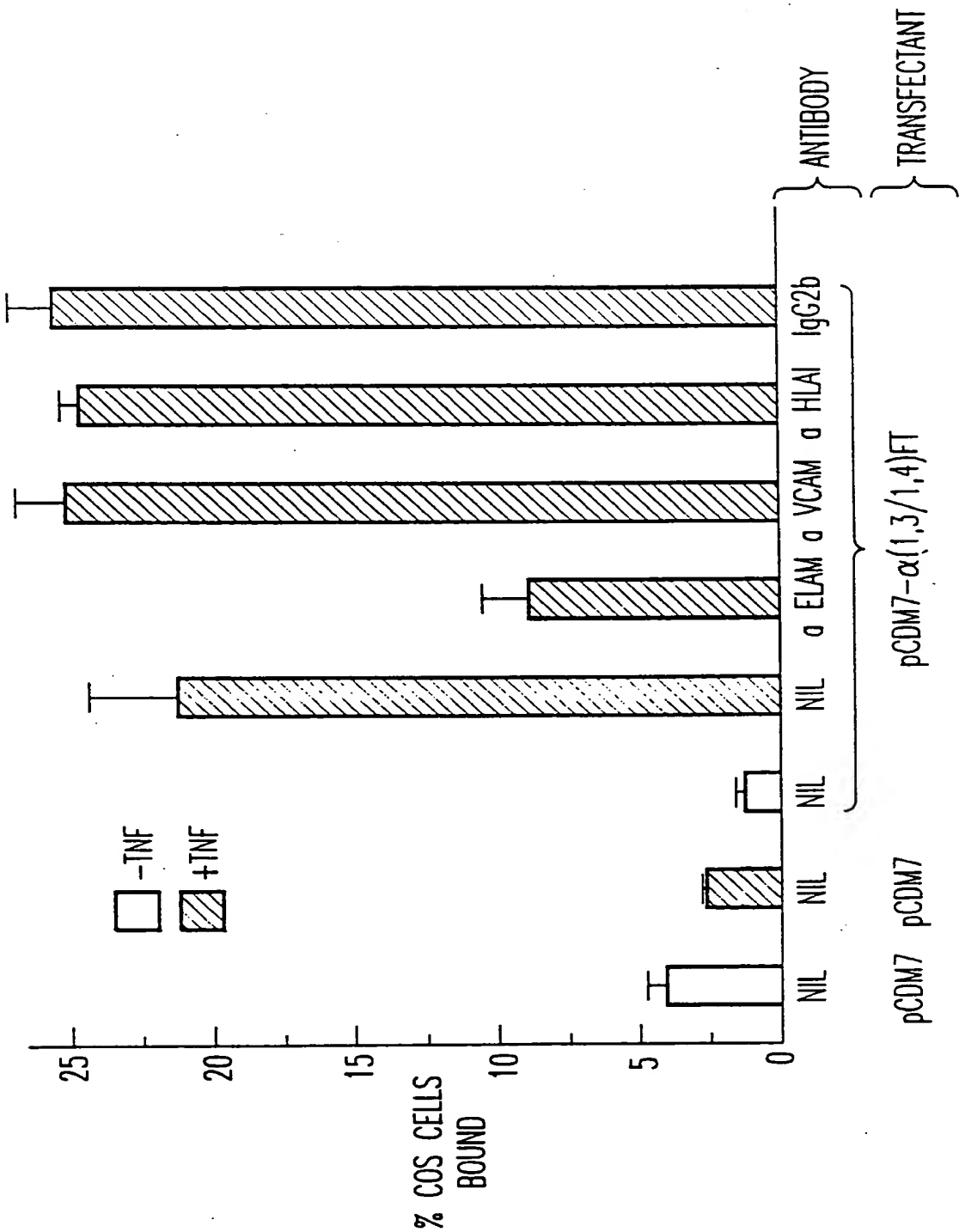
% COS CELLS BOUND
0
5
10
15
20
25
-TNF
+TNF
NIL
NIL
NIL
NIL
a ELAM
a VCAM
a HLAI
IgG2b
pCDM7
pCDM7
pCDM7-α(1,3/1,4)FT
TRANSFECTANT
ANTIBODY

FIG. 4C

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07678

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5):A61K 31/73; C07H 15/04, 5/06; C12N 5/10
U.S. CL.: 435/240.2; 536/53; 514/23, 25, 54

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/240.2; 536/53; 514/23, 25, 54

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts Service: ELAM and receptor# or ligand#

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 259, no. 14, issued 25 July 1984, **LAMBLIN ET AL.**, "Primary Structure Determination of Five Sialylated Oligosaccharides Derived from Bronchial Mucus Glycoproteins of Patients Suffering from Cystic Fibrosis", pages 9051-9058, see entire document.	1
X	CANCER RESEARCH, Vol. 44, issued November 1984, **FUKUSHIMA ET AL.**, "Characterization of Sialosylated Lewis X as a New Tumor-Associated Antigen", pages 5279-5285, see entire document.	1
X	EUROPEAN JOURNAL OF BIOCHEMISTRY, Vol. 177, no. 2, issued November 1988, **VAN HALBEEK ET AL.**, "Isolation and Structural Characterization of Low-molecular-mass Monosialyl Oligosaccharides Derived from Respiratory-mucus Glycoproteins of a Patient Suffering from Bronchiectasis", pages 443-460, see entire document.	1

(cont.)

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 January 1992	14 FEB 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Nancy S. Carson (vsh)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	CHEMISTRY AND PHYSICS OF LIPIDS, Vol. 42, issued 1986, **HAKOMORI**, "Tumor-associated Glycolipid Antigens, Their Metabolism and Organization", pages 209-233, see entire document.	1
X,P	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, (USA), Vol. 88, issued February 1991, **TIEMEYER ET AL.**, "Carbohydrate Ligands for Endothelial-leukocyte Adhesion Molecule 1", pages 1138-1142, see entire document.	1-13
X,P	BIOCHEMICAL SOCIETY TRANSACTIONS, Vol. 19, no. 3, issued August 1991, **LOWE ET AL.**, "A Transfected Human Fucosyltransferase cDNA Determines Biosynthesis of Oligosaccharide Ligand(s) for Endothelial-leukocyte Adhesion Molecule 1", pages 649-653, see entire document.	1-13
X,P	THE JOURNAL OF BIOLOGICA CHEMISTRY, Vol. 266, no. 23 issued 15 August 1991, **BERG ET AL.**, "A Carbohydrate Domain Common to both Sialyl Le A and Sialyl Le X is Recognized by the Endothelial Cell Leudocyte Adhesion Molecule ELAM-1", pages 14869-14872, see entire document.	1-13
A,P	THE JOURNAL OF CELL BIOLOGY, Vol. 115, no. 2,issued October 1991, **ZHOU ET AL.**, "The Selectin GMP-140 Binds to Sialylated, Fucosylated Lactosaminoglycans on both Myeloid and Nonmyeloid Cells", pages 557-564.	1-13
X,P	CELL, Vol. 63, issued 21 December 1990, **GOELZ ET AL.**, "ELFT: A Gene that Directs the Expression of an ELAM-1 Ligand", pages 1349-1356, see entire document.	1-13
X,P	SCIENCE, Vol. 250, issued 23 November 1990, **WALZ ET AL.**, "Recognition by ELAM-1 of the Sialyl-Le X Determinant on Myeloid and Tumor Cells", pages 1132-1135, see entire document.	1
X,P	SCIENCE, Vol. 250, issued 23 November 1990, **PHILLIPS ET AL.**, "ELAM-1 Mediates Cell Adhesion by Recognition of a Carbohydrate Ligand, Sialyl-Le X", pages 1130-1132, see entire document.	1
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA), Vol. 87, issued March 1990, **HESSION ET AL.**, "Endothelial Leukocyte Adhesion Molecule 1: Direct Expressions Cloning and Functional Interactions", pages 1673-1677, see entire document.	1-13

(cont.)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A,P	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Vol. 179, no. 2, issued 16 September 1991, **TAKADA ET AL.**, "Adhesion of Human Cancer Cells to Vascular Endothelium Mediated by a Carbohydrate Antigen, Sialyl Lewis A", pages 713-719, see entire document.	1-13
T	THE JOURNAL OF EXPERIMENTAL MEDICINE, Vol. 174, issued December 1991, **BERG ET AL.**, "The Cutaneous Lymphocyte Antigen In a Skin Lymphocyte Homing Receptor for the Vascular Lectin Endothelial Cell-Leukocyte Adhesion Molecule 1", pages 1461-1466, see entire document.	1-13

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . . . . . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.